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Brit. J. Pharmacol. (1947), **2**, 221.

THE TECHNIQUE OF TESTING CHEMOTHERAPEUTIC ACTION ON *PLASMODIUM GALLINACEUM*

BY

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From the National Institute for Medical Research, London

(Received December 21, 1945)

During the war *P. gallinaceum* and *P. lophurae* came into general use for testing the antimalarial action of new compounds. The purpose of the present work, carried out during 1943 and 1944, and withheld from publication owing to wartime restrictions, was to compare different methods of dosage in tests with *P. gallinaceum*, and to see how much the answer obtained depended upon the technique employed.

In all tests the compounds were judged by their ability to reduce parasitaemia to a low level; little or no account was taken of the power of drugs to sterilize the birds and thus to produce a radical cure of the infection.

METHODS

The general plan of the test was based on confidential information received from the American Board for the Co-ordination of Malaria Studies and from Mr. D. G. Davey, of Imperial Chemical Industries, Ltd.

The strain of *P. gallinaceum* used was obtained from the Molteno Institute, Cambridge, and was derived from the original strain introduced into Europe by Brumpt. It was maintained by blood passage in young chickens. The chickens were about 10 days old at the time of inoculation and weighed 60–80 g.; they were infected by the intravenous injection of about 10^7 parasitized cells per chick. In a typical experiment the percentage of parasitized erythrocytes, resulting from this inoculum, was 1 per cent after 2 days, 6 per cent after 3 days, 30 per cent after 4 days, and 50–90 per cent after 5 days, at which time chickens often died; if they survived, the parasites in the blood became less numerous, but many died with exoerythrocytic forms in the brain two weeks after inoculation. The drugs were given by mouth with a syringe and blunt needle. Since most drugs supplied for testing are very limited in amount it was considered that the administration of drugs in the diet (as described by Marshall *et al.*, 1942) would not be practicable. The blood of the infected chicks was examined on the 3rd and 5th days and the percentage of parasitized erythrocytes on the 5th day was determined.

To test for the prophylactic activity of drugs, the heads and thoraces of batches of mosquitoes known to contain sporozoites in their salivary glands were ground in saline and the resultant suspension centrifuged lightly to throw down the chitinous parts of the insects. The supernatant was drawn off and made up in a mixture of heparinized chick

plasma and saline, containing at least 50 per cent (v/v) plasma, so that 0.2 c.c. contained the equivalent of one infected mosquito. This suspension was used to infect chicks by intravenous inoculation of 0.2 c.c. per chick. Chicks so infected usually exhibited parasites in the peripheral blood 5–7 days after inoculation, and died 6–10 days after infection as a result of massive infection of the endothelial cells of the cerebral capillaries with exoerythrocytic forms. The drugs were administered in the manner described above, dosage being started 2 hours before infection. In both types of experiment the geometrical mean of the responses of the individual birds in a group was taken; this was compared with the geometrical mean of the responses of the group of untreated control chickens.

The following salts of quinine, mepacrine (atabrine), and pamaquin (plasmoquin) were used: quinine bisulphate containing 59 per cent anhydrous quinine, mepacrine methansulphonate (quinacrine soluble, May & Baker) containing 65 per cent of mepacrine base (3rd ADD. B.P. 1932, p. 15) and the methylene bis-hydroxynaphthoate of 6-methoxy-8-δ-diethylamino-α-methylbutyl)-aminoquinoline (pamaquin) containing 45 per cent of base (4th ADD. B.P. 1932, p. 24). All amounts of the drugs quoted refer to these salts. Sulphadiazine powder was used as the pure substance.

Measurement of the blood concentration of the drugs

An attempt was made to measure the levels of quinine, mepacrine, and sulphadiazine in the blood after the different dose schedules employed. In order to facilitate the taking of blood samples, larger chickens were used in these experiments than in the therapeutic experiments.

Quinine.—The blood level of quinine at varying periods of time after dosing was measured by a modification of the method of Kelsey and Geiling (1942), kindly devised by Prof. C. Rimington. The estimations were carried out on 0.5 c.c. blood, drawn from the heart, of 3–4 months old chickens weighing about 800–1,000 g. A series of blood samples was taken from each chicken. The oxalated blood sample was pipetted into 3.5 c.c. distilled water and the blood proteins were digested by the addition of 1 c.c. 10 per cent sodium hydroxide and heating in a water bath for 15 min. The samples were then extracted with 25 c.c. of sodium-dried ether containing 5 per cent (v/v) petrol in a 50-c.c. separating-funnel. The ether extract was washed twice with 10 c.c. $N/10$ sodium hydroxide and the quinine extracted with 4 c.c. $N/10$ aqueous sulphuric acid in three successive volumes of 1.5 c.c., 1.5 c.c., and 1 c.c. of acid. The acid extract was then made up to 5 c.c. by the further addition of sulphuric acid and its fluorescence was measured in the Rimington fluorescence comparator (Rimington, 1943). The amount of quinine present in the extracts was ascertained by comparison with a standard curve obtained by measuring the fluorescence observed when known amounts of quinine were added to blood and extracted as above.

Mepacrine.—For the estimations of mepacrine the appropriate doses were given to 800–1,000 g. chickens and blood was withdrawn from the heart. Repeated bleedings of the same chickens sometimes led to false readings of the drug level; consequently several birds were placed on the same dose schedule and no bird was bled more than three times. Coagulation of the blood was prevented by potassium oxalate; if the sample had to wait more than 24 hours before examination it was stored at −12° C. The estimations were kindly carried out for us by Major J. Reid of the Royal Army Medical College; he used a modification of Masen's method (1943).

Sulphadiazine.—Appropriate doses of sulphadiazine were given to 28-day-old chicks. 0.02 c.c. blood was taken from the leg at intervals, and the concentration of sulphadiazine therein was measured by a modification of the method of Marshall and Cutting (1938). The volume was measured in special micro-pipettes and washed out into 2.5 c.c. of acid

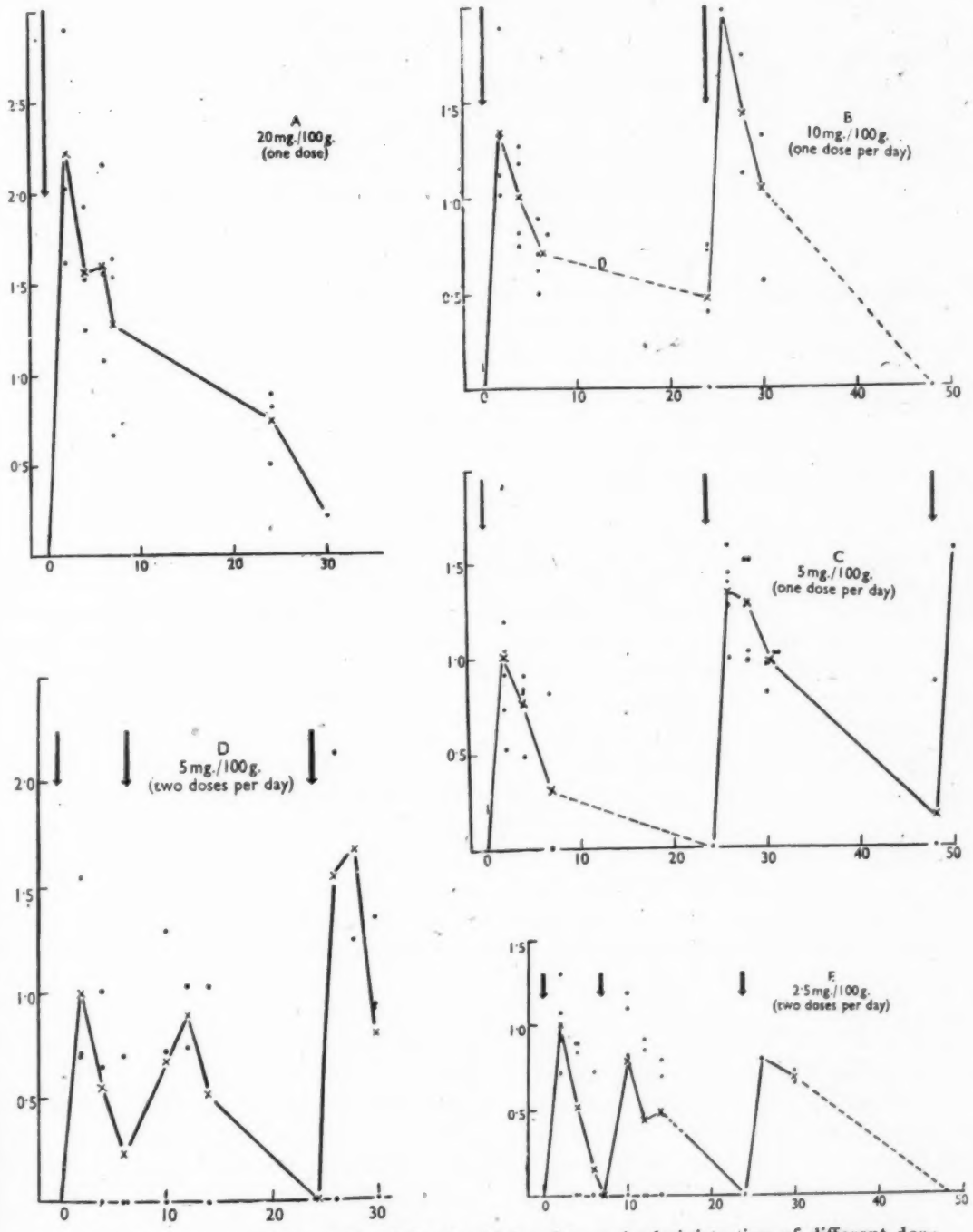

FIG. 1.—Blood concentrations of quinine in chicks after oral administration of different dose schedules of quinine bisulphate. Ordinates: blood concentrations in mg. per litre. Abscissae: hours after dosage. • = levels in individual birds. x——x = average levels. Arrows indicate dosage.

saline (0.85 per cent NaCl in *N*/50 HCl). The reagents, 0.1 c.c. 0.3 per cent sodium nitrite, 0.1 c.c. 1.5 per cent ammonium sulphamate, and 0.1 c.c. 0.1 per cent naphthyl ethylenediamine dihydrochloride, were then added to 2.5 c.c. of the supernatant, and the intensity of the fully developed colour was measured on a Spekker absorptiometer combined with a spot galvanometer.

EXPERIMENTS

TROPHOZOITE-INDUCED INFECTIONS

Effect of concentration or dispersion of the dose

The first investigations were made to determine the effect of concentrating the whole amount of antimalarial compound (quinine, mepacrine, pamaquin, or sulphadiazine) into a single administration or of dispersing it over several days in a series of smaller doses. The total amount administered remained the same, an amount being employed which was about the minimum effective level when given according to the most effective regime. A study was made of the results of the different dose schedules on (*A*) the resultant blood-concentration, (*B*) the therapeutic response.

A—Blood concentrations

Quinine.—Fig. 1 shows the mean curve for the group of birds receiving quinine; it was obtained by taking the average of the blood concentrations at each particular time. Considerable individual variation in the levels was observed in different chickens, particularly in the series 5 mg. twice daily for 2 days and 2.5 mg. twice daily for 2 days. The mean curve was only an approximate indication of what might happen in any special instance. On the whole the levels were comparable with those found by Kelsey *et al.* (1943). The absorption of quinine was rapid, the peak in the blood concentration occurring at approximately 2 hours, but the compound soon disappeared from the blood again, so that the period of antimalarial action was presumably brief in most cases. The level attained in the blood was not directly proportional to the size of the dose, since a dose of 2.5 mg. produced a peak mean blood concentration of 1.0 mg. per litre (Fig. 1E), while a dose of 20 mg. (8 times as great) produced a peak of 2.1 mg., only twice as high (Fig. 1A).

Mepacrine.—The blood concentrations of mepacrine are shown in Fig. 2, the line being drawn through the mean concentrations at the different periods of sampling. The variations between the different birds were greater than those between the different dose schedules so that from this small number of birds no reliable conclusion could be drawn. On the whole, it appeared that with mepacrine there was a peak in the blood concentration occurring about two hours after the dose, followed by a prolonged plateau at a lower level. This is well exemplified in the curve obtained after the administration of 8 mg. per 100 g. (Fig. 2A). Marshall and Dearborn (1946a) found that in the treatment of *P. lophurae* infections in ducks by mepacrine the therapeutic response was

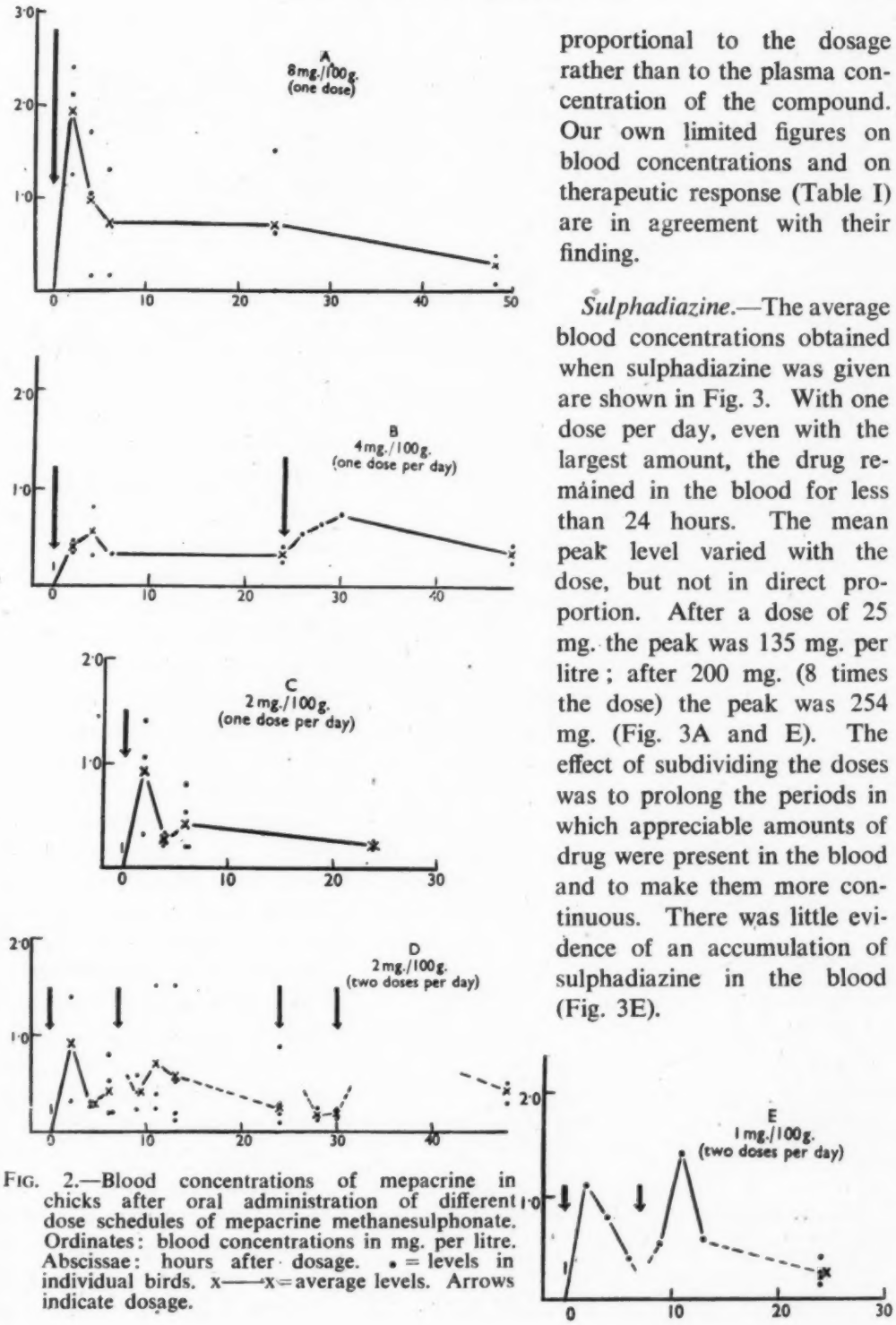

FIG. 2.—Blood concentrations of mepacrine in chicks after oral administration of different dose schedules of mepacrine methanesulphonate. Ordinates: blood concentrations in mg. per litre. Abscissae: hours after dosage. • = levels in individual birds. x——x = average levels. Arrows indicate dosage.

proportional to the dosage rather than to the plasma concentration of the compound. Our own limited figures on blood concentrations and on therapeutic response (Table I) are in agreement with their finding.

Sulphadiazine.—The average blood concentrations obtained when sulphadiazine was given are shown in Fig. 3. With one dose per day, even with the largest amount, the drug remained in the blood for less than 24 hours. The mean peak level varied with the dose, but not in direct proportion. After a dose of 25 mg. the peak was 135 mg. per litre; after 200 mg. (8 times the dose) the peak was 254 mg. (Fig. 3A and E). The effect of subdividing the doses was to prolong the periods in which appreciable amounts of drug were present in the blood and to make them more continuous. There was little evidence of an accumulation of sulphadiazine in the blood (Fig. 3E).

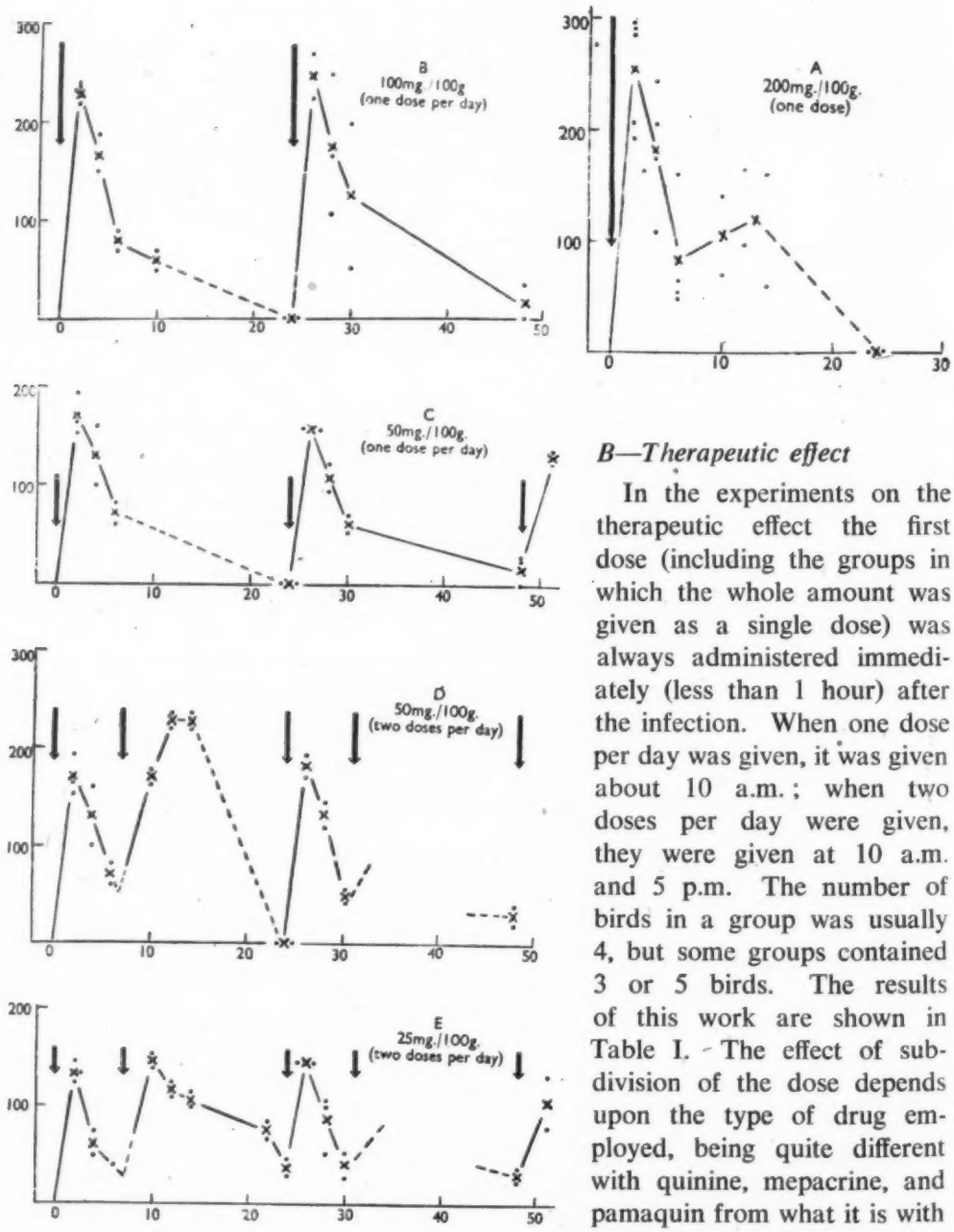

FIG. 3.—Blood concentrations of sulphadiazine in chicks after oral administration of different dose schedules of sulphadiazine powder. Ordinates: blood concentrations in mg. per litre. Abscissae: hours after dosage. • = levels in individual birds. x——x = average levels. Arrows indicate dosage.

B—Therapeutic effect

In the experiments on the therapeutic effect the first dose (including the groups in which the whole amount was given as a single dose) was always administered immediately (less than 1 hour) after the infection. When one dose per day was given, it was given about 10 a.m.; when two doses per day were given, they were given at 10 a.m. and 5 p.m. The number of birds in a group was usually 4, but some groups contained 3 or 5 birds. The results of this work are shown in Table I. The effect of subdivision of the dose depends upon the type of drug employed, being quite different with quinine, mepacrine, and pamaquin from what it is with sulphadiazine. With the former three compounds, the more the dose is concentrated the greater the response, and conversely. The drugs are

TABLE I

THE EFFECT OF DIFFERENT DOSE SCHEDULES OF STANDARD DRUGS ON THE RESPONSE OF TROPHOZOITE-INDUCED INFECTIONS

Drug	Total dose mg./100 g.	Percentage of cells parasitized on 5th day after different dose schedules					
		Total dose on 1st day	1/2 dose once daily 2 days	1/4 dose twice daily 2 days	1/4 dose once daily 4 days	1/8 dose twice daily 4 days	Untreated controls
Quinine ..	20	4.1	0.52	0.59	4.7	21.6	39.3
		1.95	1.18	1.20	24.4	17.2	53
		—	—	4.18	21.9	—	58.2
Mepacrine	8	0.61	1.30	52.3	48.9	47.9	59.5†
		0.1	0.1	1.8	3.7	—	38.5
	10	0.1	0.1	0.1	1.2	3.1	65.7
Pamaquin	0.4	—	—	5.24*	56.7	—	54.8
	0.4	0.1	—	2.9	70.9	54.7	58.3
	0.6	1.06	—	—	3.09	9.85	44
		0.1	0.1	1.35	17.4	2.51	63.9
	1	0.1	0.1	0.1	0.1	1.2	41.4
Sulphadiazine ..	200	37	28.8	38.7	12.6	0.12	39.5

* Poor test. Only 1 chick remained alive. † 6th day count.

most effective if given in a single large dose or if the dose is subdivided only into two ; as the subdivisions get more numerous, less antimalarial effect is shown. Apparently the action of these drugs upon the parasites is rapid, a short interval of time being sufficient for it to take place, but on the other hand it is strongly effective only if a certain level of blood concentration is reached—e.g., with quinine, a level of over 1 μg. per c.c. ; this critical level of concentration is presumably not reached when the dose of drug administered falls below a certain quantity, even if the dose is repeated twice daily for four days. With sulphadiazine on the other hand, the greatest antimalarial effect is obtained when the administration is spread over the whole four days, subdividing the amount into 8 small doses. The administration of the drug in 1–4 doses concentrated into the first two days is non-effective. Even the slight modification of giving a single dose on each of four days, instead of two smaller doses on each of these days, greatly diminishes the antimalarial effect. This class of compound presumably exerts a slower action upon the parasites than quinine, mepacrine, or pamaquin do ; and the duration of action is more important than the concentration over any particular short period.

Effect of time of starting the treatment

It has been reported from America that some workers begin treatment several hours before infecting the birds, others wait until after the inoculation to begin treatment. The latter procedure is the more convenient in practice as the whole

batch of chicks can be inoculated without the necessity of identifying each individual in the process; moreover, birds in which the inoculation has been unsatisfactory in any way can easily be discarded and replaced by new ones. An investigation was made in order to discover whether this difference of procedure affected the therapeutic response observed, and the results are shown in Table II. As will be seen, there was no significant difference in the response whether the first dose was given immediately after inoculation, or 5 hours before inoculation. Accordingly, in our routine testing of drugs the first dose is always given almost immediately (less than 1 hour) after inoculation.

TABLE II

THE EFFECT OF THE TIME OF STARTING DOSAGE ON THE RESPONSE OF TROPHOZOITE-INDUCED INFECTIONS

Drug	Total dose mg./100 g.	Dose given twice daily for 14 days	Percentage of cells parasitized on 5th day when dosage started		Controls
			immediately after infection	5 hours before infection	
Quinine ..	20	2.5 mg.	10.7	19	38.5
			3.02	14.8	41.4
			29	24.9	61.1
	24	3 mg.	6	7.3	44
Mepacrine ..	10	1.25 mg.	28.3	21.5	41.4
			0.96	1.16	44
			9.01	5.52	61.1
Pamaquin ..	0.4	0.05 mg.	45.9	39.2	38.5
	0.6	0.075 mg.	21.1	28.1	44
			7.94	12	63.9
	1	0.125 mg.	1.2	4.59	41.4
Sulphadiazine	200	25 mg.	0.42	0.8*	39.5

Each of the groups of treated chicks contained 4 birds; the groups of controls usually contained 6–10 birds.

* Started 2 hours before infection in this case as sulphadiazine reaches its peak in the blood 2 hours after oral administration.

A further study of the effect of delay in commencing treatment was made by giving the same total amount of mepacrine either all on the last (3rd) day after inoculation or spread out over the previous days. This is a reverse of the dose schedules described in Table I. The results are shown in Table III. The best therapeutic responses were obtained when the drug is concentrated on to the last two days of the treatment. Probably in this experiment the effect of concentrating the dosage was more important than the time of starting it. (On this occasion mepacrine seems to have been less effective than in the experiments of Table I.)

TABLE III

THE EFFECT OF DELAY IN COMMENCING TREATMENT WITH MEPACRINE

Drug	Schedule	% cells parasitized on 5th day	Control
Mepacrine	6 mg. twice daily on the 4th day	2.62	65.7
	3 mg. ,, ,, ,, ,, 3rd and 4th days ..	0.56	
	2 mg. ,, ,, ,, ,, 2nd, 3rd, and 4th days ..	18.7	
	1.5 mg. ,, ,, ,, ,, 1st, 2nd, 3rd, and 4th days	35.2	

In each group the total dose was 12 mg. per 100 g.
Each group contained 4 chicks.

Sporozoite-induced Infections

Similar investigations about the effect of concentrating or dispersing the treatment were made with sporozoite-induced infections—i.e., the prophylactic action of the compounds upon the pre-endoerythrocytic forms of the parasites was studied instead of the therapeutic action upon the endoerythrocytic ones (trophozoites, schizonts, etc.). At the time of conducting this work the only compounds known to have a prophylactic action were the sulphonamides, and accordingly the investigations were made using sulphadiazine. Two types of experiment were made. In the first, a given amount of sulphadiazine (200 mg. per 100 g.) was either concentrated at the beginning of the infection or dispersed over the first four days ; in the second, treatment was more intense (total 800 mg. per 100 g.) and it was given either in the first two days or on the 3rd and 4th days after infection. The results of both these investigations are shown in Table IV.

In the first type of experiment the data show that the more the treatment was spread out over the first four days the more effective it was ; treatment restricted to the first day had little or no effect, even if the total dosage was doubled (i.e., total dose 400 mg.). These results with pre-endoerythrocytic stages of the parasite agreed with those obtained concerning the action of sulphadiazine upon the endoerythrocytic forms ; they support the view that the antimalarial action of sulphadiazine is of such a type that duration of exposure is more important than intensity.

The second part of these experiments concerns the sensitivity of the different stages of the parasite to sulphadiazine. Treatment restricted to the first day had little or no action ; apparently the sporozoites in their original form or in their early development after inoculation were not much affected by the compound during a single day. Treatment given on the 3rd and 4th days was slightly more effective in delaying the development of patent infection than that given on the 1st and 2nd days ; but much of this effect was due to the postponement of treatment, and if the interval is measured between the onset of the patent infection and the end of treatment, there was little real difference between the degree of parasitaemia observed in the two groups. However, ultimate survival was much

TABLE IV

EFFECT OF DIFFERENT DOSE SCHEDULES AND OF TIME OF DOSAGE OF SULPHADIAZINE ON SPOROZOITE INFECTIONS

	Total dose mg./100 g.	Individual doses per 100 g.	Days of treatment	Percentage of cells parasitized on day						Remarks
				8	9	10	11	12	13	
First part of experiment	200	*200 mg. once daily	1st		24.6 2 D EEF+		23 1 D EEF+			
	200	100 mg. once daily	1st, 2nd		1.2	2 D EEF+	49.3 2 D			
	200	50 mg. once daily	1st, 2nd, 3rd, 4th		Less than 0.1		1.6		14.1 2 D EEF+	2 chicks died on 17th day
	200	*50 mg. twice daily	1st, 2nd		Less than 0.1		0.66		14.9	3 chicks died on 15th day. EEF+
	200	*25 mg. twice daily	1st, 2nd, 3rd, 4th		Less than 0.1		Less than 0.1			On 15th day: 51.2. 1 D. EEF+. 1 chick died on 16th day. 1 chick survived
	Control	*—	—		45.5 2 D		1 D			
	200	*200 mg.	1st	10.2	1 D	48 2 D EEF+	1 D			
	200	25 mg. twice daily	1st, 2nd, 3rd, 4th	Less than 0.1		Less than 0.1	0.4			2 chicks failed to show parasitaemia. 2 chicks died on 17th day. EEF+
	Control	—	—	8.03 1 D	1 D EEF+	32.2	2 D			
Second part	800	200 mg. twice daily	1st, 2nd	Less than 0.1		1.2		6.9	2 D	2 chicks died on 14th day. EEF+
	400	200 mg. twice daily	1st	4.3	1 D EEF+	11.6	1 D	1 D		1 chick died on 14th day
	Control	*—	—		45.5 2 D		1 D			
	800	*200 mg. twice daily	1st, 2nd	Less than 0.1		3.1	1 D EEF+		42.5 2 D	
	800	200 mg. twice daily	3rd, 4th	Less than 0.1		Less than 0.1			24.3	1 chick died on 17th day. EEF+. 3 chicks survived
	Control	—	—	8.03 1 D	1 D EEF+	32.2	2 D			

The inoculum used in these experiments contained the approximate equivalent of 1 infected mosquito per chick. The groups marked with an asterisk contained 3 chicks, the others contained 4.
The "1st day" dose was given 2 hours before infection with sporozoites. D, Chick died.
EEF+ Exoerythrocytic forms found in the brain post mortem.

better in the group treated on the 3rd and 4th days. It is noteworthy that (with the possible exception of 2 chicks) sulphadiazine in these doses failed to sterilize any of the birds ; it only delayed the multiplication of the parasites for a shorter or longer period. In this respect its action upon pre-endoerythrocytic forms was indistinguishable from that upon endoerythrocytic forms. The results thus suggest that the antimalarial action of sulphadiazine is exerted both on pre-endoerythrocytic forms of *P. gallinaceum* (cryptozoites, etc.) and on endoerythrocytic forms (trophozoites and schizonts) ; that this action tends to be plasmodiostatic rather than plasmodiocidal ; and that its effectiveness depends upon the duration of exposure rather than upon the intensity.

Discussion

It has been shown that under the experimental conditions described the maximum effect of a given small quantity of quinine, mepacrine, or pamaquin upon the trophozoites is obtained when the treatment is concentrated into one or two days. The greatest effect of a given quantity of sulphadiazine upon either trophozoites or upon pre-endoerythrocytic forms is obtained when the treatment is spread out over the whole period of four days. With the former drugs, intensity of action seems more important than duration ; with sulphadiazine, duration is more important than intensity. In the treatment of trophozoite-induced infections there is no significant difference in the response observed whether the first dose is given 5 hours before inoculation or immediately after it. The action of sulphadiazine is exerted both on pre-endoerythrocytic forms of *P. gallinaceum* and on endoerythrocytic ones.

A comparison has been made by Marshall and Dearborn (1946b) of the single-daily-dose and of the drug-diet methods of treatment in bird-malaria (*P. lophurae* in ducks) in relation to the therapeutic activity of numerous compounds ; as found above, they showed that the relative activities of the different compounds are considerably affected by the concentration or dispersion of the dosage.

These results may now be considered in relation to the devising of routine tests of the antimalarial action of new compounds. If the compound were given in a single dose (as is customary in experiments with mouse trypanosomiasis) the action of quinine, etc., would be manifested while that of sulphadiazine would probably be missed. The maximum dose which can be tolerated depends upon the toxicity of the compound, and this is usually such that a series of doses spread over four days is much less injurious than a large dose on a single occasion. Accordingly the usually accepted regime of treatment spread over four days has much to recommend it since (1) it provides sufficient duration for the slower acting compounds such as sulphadiazine, (2) it minimizes toxicity and allows larger amounts of the compound to be given, and (3) it is not unduly laborious to administer. No experiments have been made in this work using schedules extending over more than four days ; as a routine measure such schedules would appear

to be more wasteful of labour and of material (which is often scarce) while offering insufficient compensating advantage. The routine tests carried out in this laboratory are therefore based on treatment lasting four days. The general procedure has been described above in the section upon methods. For infections induced by trophozoites the response is read as the percentage of parasitized erythrocytes on the fifth day, and the geometrical mean for the group of birds is compared with that for the group of untreated controls. If a compound is found to be active in the maximum tolerated dose, the dose is reduced in subsequent tests until the lowest dose is found which reduces the percentage of parasitized cells to about one or two. This is considered to be the approximate minimum effective (therapeutic) dose. For infections induced by sporozoites, the chief criteria of the activity of a drug are complete suppression of infection or a delay in the appearance of parasites in the peripheral circulation, and prolongation of the life of the chicken or recovery from the infection. For quantitative determination of the activity, measurement is made of the minimum effective (prophylactic) dose which prevents the percentage of parasitized cells being greater than 1–2 on the 7th to 9th day when the infection has reached a high level in the controls; no compound tested to date in this laboratory has been sufficiently effective for its activity to be expressed as the minimum dose which completely prevents infection. The antimalarial activity, therapeutic or prophylactic, as expressed by these minimum effective doses, is compared with the toxicity as determined by the maximum tolerated dose for mice, when administered orally twice daily for four days. Since the drugs are designed for use in man, the toxicity for mammals is more significant than that for birds, even though the tests for antimalarial potency are carried out in chickens.

Summary

The methods used in the authors' laboratory for testing the antimalarial action of drugs upon infections of *P. gallinaceum* induced by trophozoites and sporozoites are described.

A given amount of quinine, mepacrine, and pamaquin exerts the maximum effect on trophozoite-induced infections of *P. gallinaceum* if it is concentrated into the first day or first two days of treatment; with these compounds intensity of action is more important than duration. A given amount of sulphadiazine produces the maximum effect upon trophozoite- or sporozoite-induced infections if it is dispersed over all the four days of treatment; with this compound duration of action is more important than intensity. The action of sulphadiazine is exerted both on the pre-endoerythrocytic forms of *P. gallinaceum* (cryptozoites, etc.) and on the endoerythrocytic forms (trophozoites, etc.).

In the test on trophozoite-induced infections there is no significant difference in the response whether the first dose is given immediately after the inoculation or 5 hours before it.

The blood concentration curves of quinine, mepacrine, and sulphadiazine on the different dose schedules were determined. Increasing the dose eight times increases the peak concentration in the blood only about twice.

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THE TOXICITY OF ALKYL FLUOROPHOSPHONATES IN MAN AND ANIMALS

BY

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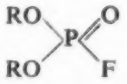
In May, 1940, we prepared the dimethyl and diethyl esters of fluorophosphonic acid and tested their effects on animals as lethal inhalants. When, about a year later, McCombie and Saunders prepared the di*iso*propyl ester, we found it had an even more powerful effect than the dimethyl and diethyl compounds.



$R = CH_3$, C_2H_5 or $(CH_3)_2CH$

dialkyl fluorophosphonate

The present paper deals with this early and first systematic study of the toxicity of the fluorophosphonates which are now widely used for physiological experiments and clinical trials. When the toxic action was discovered it was not possible for us to publish the results, which were, however, circulated as a report to the Ministry of Supply. (Adrian, Kilby, and Kilby, 1940.)

The preparation of the fluorophosphonates and this study of their toxic properties was prompted by an observation of Lange and Krueger (1932). They had prepared the dimethyl and diethyl compounds and had stated at the end of their communication that inhalation for a few minutes might lead to difficulty in breathing, to disturbance of vision, hypersensitivity to light, and even to loss of consciousness.

METHODS

The dimethyl and diethyl fluorophosphonates used in these experiments were prepared by the method described by Lange and Krueger, and the di*iso*propyl ester was kindly supplied by Dr. H. McCombie, Dr. B. C. Saunders and their research team, who made it by the method announced in a preliminary communication. (McCombie and Saunders, 1946.)

The action and toxicity of fluorophosphonates were studied in man by inhalation and in animals by inhalation as well as by intravenous or subcutaneous injection.

Inhalation in man

An approximately cubical steel-framed glass chamber of 10 cu.m. capacity was used. The desired concentration of ester vapour was obtained by dissolving the calculated

weight of the compound in about 20 c.c. of ether and spraying it into the chamber by means of an atomizer worked by compressed air, mixing being achieved by three electric fans. After about 30 sec., the subjects, one to four in number, who had been waiting in an air-lock, walked into the chamber and quickly shut the door behind them. At the end of the exposure, the subjects left through the air-lock, and the chamber was cleared by a large suction fan. In the calculation of the concentration (e.g. 1 in 100,000) it is assumed that the ester is completely volatilized and that the gram-molecular weight occupies a volume of 22.4 litres.

Inhalation in animals

Static method.—A wood-framed glass chamber of 1.78 cu.m. capacity was used. A batch of animals (e.g., 3 rabbits, 4 guinea-pigs, 6 rats, and 10 mice) was placed inside in wire cages, the chamber sealed and the calculated amount of fluorophosphonate dissolved in 20 c.c. of ether sprayed into it. The exposure was timed from when about half of the material had been dispersed, an operation which usually took less than a minute. Mixing was achieved by an electric fan in the chamber. At the end of the exposure, an exhausting fan was switched on, the chamber opened and the cages removed by two operators wearing service respirators. The animals were removed to another room and kept under observation. Post-mortem examinations were made on animals that died.

Constant-flow technique.—An apparatus was constructed containing an all-glass exposure chamber of about a litre capacity fitted with ground glass joints, in which either one rat or four mice could be placed. A constant stream of air, at a known rate of flow, was passed through this chamber, either by using compressed air which was passed through a gas-meter in series with the chamber or, in other experiments, by suction, measured amounts of water being run out of a 20-litre aspirator. All or part of the air stream could be passed through a bubbler containing di*iso*propyl fluorophosphonate, which was weighed before and after the experiment to determine the amount of ester volatilized. The rate of volatilization was also controlled by varying the temperature of the water-bath in which the bubbler was immersed. An identical bubbler containing sulphuric acid was fitted in parallel, and in an exposure pure air was passed through the chamber until conditions were steady and the animal calm, and then by means of a three-way tap the air stream was caused to pass through the ester for the desired time. In this manner it was possible to make 1-min. exposures, because the desired concentration was rapidly attained at the beginning of the exposure, and the ester quickly swept out at the end. When only a part of the total air stream was passed through the bubblers, flow-meters were included in the circuit so that the air streams could be adjusted to give approximately the desired concentration of ester. The two air streams were allowed to mix in a suitable compartment before entering the exposure chamber. The bubblers, mixers and exposure chamber were connected by ground glass joints. When necessary, the gases leaving the exposure chamber were passed through towers containing activated charcoal or through service respirator canisters in order to remove the ester vapours.

RESULTS

Inhalation in man

The inhalation of air containing low concentrations of dimethyl, diethyl, or di*iso*propyl fluorophosphonate leads to effects qualitatively similar for all three esters, but varying in intensity with the nature of the ester dispersed, its concentration, and the time of exposure. Within a minute or so a feeling of tightness of the throat is noticed and respiration becomes slightly more laboured.

A few minutes later the pupils constrict to pin-point size and remain so for a long period; this causes the subject to experience the sensation that the room has dimmed, as though the sky had suddenly become heavily overcast. After a few hours, reading becomes almost impossible unless the book is held only a few inches from the eyes; at a distance of about 18 in. print appears blurred. This effect must be due to a strong constriction of the ciliary muscles. The di*iso*propyl ester has the most powerful action of the three compounds. When two subjects were exposed for 3 min. to a nominal concentration of 1 in 100,000 (82 mg./cu.m.) of this material, a tightness of the throat and slight difficulty in inspiration occurred during the exposure, and about 10 min. later the pupils constricted to pin-point size and remained so for days. The miosis subsided after about a week in the younger subject (28 years) and after two or three days in the elder (60 years). After about 24 hours there was eye-ache and headache which persisted for a day or two.

Inhalation in animals

Animals were exposed for longer periods and to higher concentrations than those used with man, and severe symptoms were produced, death frequently resulting. The effects were approximately the same in all species examined and with each of the three compounds. There was excessive salivation, nasal discharge, lacrimation and frequently pupil constriction, respiratory distress accompanied by intense gasping movements, and, in severely affected animals, convulsions leading to death, which usually occurred within about half an hour of the beginning of the 10-min. exposure, and sometimes actually during the exposure. The rapidity of action of these materials as lethal inhalants is noteworthy and the majority of animals that survived half an hour usually made a complete recovery, although a few deaths occurred up to 24 hours.

The mortalities produced in rabbits, guinea-pigs, rats, and mice by 10-min. exposures to various nominal concentrations of the three compounds are shown in Table I. For each experiment 3 rabbits, 4 guinea-pigs, 4 or 6 rats, and 4 or 10 mice were exposed in a static chamber. Of the four animal species, it will be seen that rabbits are the least and mice the most susceptible.

TABLE I

DEATHS RESULTING FROM 10-MIN. EXPOSURE OF SMALL ANIMALS TO VARIOUS CONCENTRATIONS OF FLUOROPHOSPHONIC ESTERS

Compound:	Methyl ester			Ethyl ester			*Iso*propyl ester	
Concentration:	1/5,000	1/10,000	1/20,000	1/5,000	1/10,000	1/20,000	1/10,000	1/20,000
Rabbits	0/3	0/3	0/3	0/3	0/3	0/3	2/3	0/3
Guinea-pigs ..	3/4	0/4	0/4	2/4	0/4	0/4	0/4	0/4
Rats	4/4	4/6	1/4	4/4	0/6	1/4	6/6	4/6
Mice	4/4	10/10	4/4	4/4	10/10	0/4	10/10	3/10

A more detailed study was made of the toxicity of the di*iso*propyl ester for rats and mice; the concentration necessary to kill 50 per cent of the animals exposed (LC50) was determined for each of five constant exposure times (1, 2, 5, 10, and 30 min.) by the constant-flow technique. A convenient measure of the toxicity of a lethal inhalant is obtained by the product of the LC50 and the time of exposure in minutes (*t*). This value is referred to in the present paper as the LC*t*50. Ideally it should be a constant and independent of the exposure time, but if detoxification takes place during exposure LC*t*50 should increase greatly during long exposure to a low concentration; this is observed, for instance, when hydrogen cyanide is used as a lethal inhalant. The small changes observed in the values of LC*t*50 in our experiments with fluorophosphonates, however, were probably not statistically significant, but a minimum value of the LC*t*50 at 5–10 min. might be indicated. With the static method an increase in LC*t*50 with longer exposures may indicate that the concentration of toxic agent is falling off, but this effect can be eliminated by the constant-flow technique, in which a constant concentration is maintained by replacement. The LC*t*50 values for rats using 10- and 30-min. exposures were determined by both static and constant-flow methods, and almost identical values were obtained, indicating that no appreciable decay of concentration occurred in the static method up to 30 min., so that the nominal concentrations employed in the static method used to obtain the data in Table I are probably fairly close to the true concentration.

Estimates of the LC50 and LC*t*50 values for rats and mice are shown in Table II. These estimates were obtained, by graphical interpolation, from the results of experiments in which at least 4 animals were used to determine each point on the mortality–concentration curves; in all, 197 rats and 390 mice were used.

TABLE II

LC50 AND LC*t*50 VALUES FOR RATS AND MICE EXPOSED TO DI*iso*PROPYL FLUOROPHOSPHONATE. CONSTANT-FLOW TECHNIQUE

Animal	Exposure time min.	Deaths within 2 hr.		Deaths within 48 hr.	
		LC50 mg./cu.m.	LC*t*50 mg./cu.m./min.	LC50 mg./cu.m.	LC*t*50 mg./cu.m./min.
Rats ..	1	4,200	4,200	4,200	4,200
	2	2,000	4,000	1,800	3,600
	5	700	3,500	570	2,850
	10	360	3,600	280	2,800
	30	180	5,400	150	4,500
Mice ..	1	5,000	5,000	4,000	4,000
	2	2,650	5,300	1,900	3,800
	5	750	3,750	540	2,700
	10	440	4,400	350	3,500
	30	185	5,550	150	4,500

A more detailed analysis of the survival period of these animals shows that 55 per cent of the rats and 53 per cent of the mice had died within the first 2 hours after exposure; during the next 48 hours a further 15 per cent of the rats and 22 per cent of the mice died; there were no further deaths among the rats and only 1 per cent among the mice, 30 per cent of the rats and 23 per cent of the mice surviving. The rapid lethal action of fluorophosphonates becomes even more evident if the deaths occurring during the first two hours are grouped in half-hour intervals; if for this calculation the deaths after 30-min. exposure are omitted, it is found that the majority of animals died within the first half-hour after exposure. Of the 108 rats dying within the first two hours, 95 died within the first, 10 within the second, and 3 within the third half-hour period after the beginning of exposure; the corresponding figures for 207 mice dying during the first two hours were 183, 10, 9, and 8 mice respectively for the four half-hour intervals.

Injection into animals

After injection of a solution of the di*iso*propyl ester in normal saline into the ear vein of rabbits there was excessive salivation, muscular twitchings, and loss of muscular co-ordination, sometimes urination and defaecation and usually convulsions prior to death. The pulse-rate was slowed like the respiratory-rate, but respiration ceased before the heart stopped beating. There was constriction of the pupils, commencing 2–5 min. after injection. The LD50 determined in a small number of rabbits was 0.5–0.75 mg./kg.

The LD50 for mice for subcutaneous injection of the di*iso*propyl ester dissolved in normal saline was 4 mg./kg., determined by graphical interpolation of the results of injections into batches of mice over a suitable dose range.

Atropine

Since it had been found (see Adrian, Feldberg and Kilby, 1946, 1947) that fluorophosphonates were extremely strong inhibitors of cholinesterase, experiments were made to determine whether atropine would be an effective antidote.

In rabbits intravenous injections of atropine were made either before or immediately after intravenous injection of a lethal dose of di*iso*propyl fluorophosphonate. When atropine was injected after the ester it was incapable of saving life, but when it was injected before the ester it appeared to reduce the death rate. For instance, when atropine (in doses between 4 and 50 mg./kg.) was injected immediately after a lethal dose of di*iso*propyl fluorophosphonate (1 mg./kg.) the sole effect was to alleviate the severity of the symptoms and postpone death for a short time. This effect of atropine occurred only if the atropine was given immediately after the fluorophosphonate; if given only a few minutes later atropine had no alleviating effects whatever. When, on the other hand, atropine (10 mg. per kg.) was given intravenously 10 min. before a lethal intravenous dose of fluorophosphonate (1 mg./kg.) the appearance of symptoms was not only delayed and their severity reduced, but the lives of some of the animals were actually saved; for instance, out of five rabbits so treated, three survived and the other two died only after 1½ to 3½ days, whereas all five control rabbits, given the fluorophosphonate without atropine, died within 45 min.

Discussion

The toxic effects of the fluorophosphonates resemble in many respects those of eserine and prostigmine and are probably due to the anti-cholinesterase activity which these esters have been shown to exhibit. (Adrian, Feldberg, and Kilby, 1946, 1947.) Parasympathomimetic effects are very pronounced: the eye effects, the excessive salivation and lacrimation, and the slowing of the heart may easily be explained as being due to accumulation of acetylcholine released from the parasympathetic endings. It is thus not surprising that these effects are alleviated by atropine. In addition, the fluorophosphonates appear to have a definite " nicotine-like " action on skeletal muscle and on the central nervous system. The excitatory effects of fluorophosphonates on these structures are less pronounced, and the paralysing effects predominate. The fluorophosphonates share this predominance of a paralysing action with other anti-cholinesterases, and the problem of why under certain conditions some inhibitors of cholinesterase are mainly excitatory and others mainly depressant has never been satisfactorily explained. (For review see Feldberg, 1945.)

Death from fluorophosphonate poisoning probably results from respiratory failure, partly owing to obstruction of bronchioles, but mainly to paralysis of the respiratory centre ; in this way, too, the fluorophosphonates resemble other anti-cholinesterases. However, no detailed analysis has been made of the exact nature of the cause of death.

Summary

1. Inhalation of fluorophosphonates in man and animals leads to respiratory distress, pupillary constriction, and spasm of accommodation. In animals, inhalation in higher concentrations than those used in man causes in addition excessive salivation, lacrimation, convulsions, and death, probably owing to respiratory failure. Similar effects are observed in animals on intravenous injection of fluorophosphonates.

2. The percentage of deaths among small animals after 10 min. exposure to various concentrations of dimethyl, diethyl, and di*iso*propyl fluorophosphonates is recorded. The concentration of di*iso*propyl fluorophosphonate which will kill 50 per cent of rats or mice exposed (LC50) was found for 1, 2, 5, 10, and 30 min. exposures. The product of the LC50 and the time of exposure in minutes (the LC*t*50) was found to vary between 2,700 and 4,500 mg./cu.m./min. The LC*t*50 showed a minimum for 5 to 10 min. exposures.

3. Death, if it occurred, usually took place within the first half-hour after exposure.

4. The LD50 of di*iso*propyl fluorophosphonate is about 4 mg./kg. for subcutaneous injection in mice and 0.5–0.75 mg./kg. for intravenous injection in rabbits.

5. Atropine given before the fluorophosphonate alleviated the severity of symptoms and reduced the death rate, but had little effect if given afterwards.

We are indebted to Professor E. D. Adrian and Dr. W. Feldberg for much helpful advice and criticism during this work, and to the Chief Scientist, Ministry of Supply, for permission to publish these results.

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THE MODE OF ACTION OF MYANESIN

BY

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Berger and Bradley (1946, 1947) have shown that α: β-dihydroxy-γ-(2-methylphenoxy)-propane ("myanesin") produces muscular relaxation and paralysis in experimental animals. The effects of the drug are of particular interest because paralysing doses of myanesin do not cause arrest of respiration. In this respect myanesin differs from curare and similar muscle-relaxing agents, which do not produce paralysis without simultaneous respiratory depression or arrest. It was therefore of interest to examine in greater detail the mechanism by which the effects of myanesin are produced. This report describes certain aspects of the action of the drug on voluntary muscle, the myoneural junction, peripheral nerves, and the central nervous system.

Action on the isolated voluntary muscle

The action of myanesin was investigated on the isolated rectus abdominis of the frog (*R. temporaria*) during November and December. The muscle was suspended in oxygenated Ringer's solution and arranged for recording the contractions on a kymograph. Concentrations of drugs were expressed as final concentrations in contact with the muscle. Myanesin at 1 in 750 or higher dilutions did not cause any effects. Stronger solutions such as 1 in 500 produced a slow contracture, which began about 10 minutes after the addition of the drug and gradually increased until about 1 hour later the muscle had shortened by about one quarter of the maximum possible contraction. At this stage the muscle still responded to electrical stimulation.

The effect of myanesin on the contraction produced by acetylcholine was also investigated. Acetylcholine, 1 : 100,000, produced almost maximum contraction; after 2 similar contractions had been produced, myanesin or other drugs were added and allowed to remain in contact with the muscle for 10 minutes. The muscle was then washed and the response to acetylcholine retested at suitable intervals until contractions were obtained similar to those before application of the drug.

Table I gives the reduction of the contraction expressed as the percentage of the contraction before administration of the drug. Myanesin 1 in 1,000 reduced the response to acetylcholine to about one half and 1 in 750 to about one third. Stronger solutions of myanesin were not used because they affected the muscle itself. Tubocurarine chloride and procaine hydrochloride, when similarly examined, were found to exert a very much stronger effect than myanesin. The

TABLE I

THE ANTAGONISTIC EFFECT OF MYANESIN AND TUBOCURARINE ON CONTRACTIONS OF THE FROG'S RECTUS ABDOMINIS

Contractions were produced by acetylcholine 1 in 100,000

Drug	Dilution	Reduction of contraction as per cent of original contraction
Myanesin	1 : 2,000	25
	1 : 1,000	53
	1 : 750	68
Tubocurarine chloride	1 : 1,000,000	36
	1 : 750,000	46
	1 : 500,000	60
	1 : 100,000	93

concentrations of tubocurarine chloride, procaine, and myanesin causing approximately 50 per cent reduction in the response to acetylcholine were 1 in 700,000, 1 in 6,000, and 1 in 1,000 respectively.

The experiments show that myanesin in high dilution does not antagonize the action of acetylcholine on voluntary muscle. It is therefore likely that myanesin produces its effects by a mechanism different from that of curare. Although myanesin and procaine are local anaesthetics of almost equal potency, procaine antagonizes the effects of acetylcholine more strongly than myanesin. This observation suggests that the curare-like action of local anaesthetics is an independent property of such drugs and may have little relation to their paralysing effects on nerve endings or nerve trunks.

Effect on peripheral nerves

The action of myanesin on the motor nerve was examined on a simple muscle nerve preparation of the frog. The sciatic nerve was immersed in a solution of the drug. Excitability was tested at the cut end of the nerve at 1 minute intervals with galvanic current from an induction coil. The time after which contraction of the muscle was abolished was noted. The action on sensory nerves was examined by the plexus anaesthesia method in frogs and the intracutaneous weal method in guinea-pigs as described by Bülbring and Wajda (1945). Cocaine or procaine were used as standards of comparison. All drugs were used at three dose levels.

TABLE II

LOCAL ANAESTHETIC POTENCY OF MYANESIN EXPRESSED IN TERMS OF PROCAINE AND COCAINE

Method	Number of animals used with myanesin	Number of animals used with procaine	Potency as percentage of that of procaine	Number of animals used with cocaine	Potency as percentage of that of cocaine
Motor nerve (frog)	12	12	98	12	35
Sensory nerve (frog)	12	12	96	12	39
Intracutaneous weal (guinea-pig)	18	18	69	—	—

The results are summarized in Table II. Myanesin had about the same potency as procaine and one third that of cocaine when tested on the motor and sensory nerve of the frog. When examined by the intracutaneous weal method in guinea-pigs, myanesin had about two thirds of the activity of procaine. The relatively weak local anaesthetic action of myanesin suggests that the paralysing effect of the drug is not due to a direct action on peripheral nerves.

The curare-like action

It has been shown previously that myanesin in large and nearly lethal doses can produce paralysis of the muscle to indirect but not to direct stimulation. This effect may be due either to a curare-like action at the myoneural junction or to a more central paralysis of the nerve-endings (Berger and Bradley, 1946). Further experiments were carried out to ascertain the importance of this effect in myanesin paralysis.

(*a*) *Experiments on cats.*—Chloralosed or decerebrated cats were arranged for registration of contractions of the gastrocnemius muscle. The muscle was stimulated alternately directly and indirectly at 10 seconds intervals by single induction shocks. Intravenous injection of myanesin in doses up to 150 mg. did not effect the response to direct or indirect stimulation in any way. Tubocurarine chloride (0.5 mg.) abolished the response of the muscle to indirect stimulation but hardly influenced the response to direct stimulation. The experiments show that myanesin injected into cats in doses of 150 mg. does not possess curare-like action.

(*b*) *Experiments on mice.*—White mice weighing 18 to 22 grams were injected intraperitoneally with myanesin, cocaine, or procaine. After 10 minutes they were anaesthetized with ether and decerebrated. The sciatic nerve was then exposed in the gluteal region and cut. Fifteen and thirty minutes after injection of the drug, the peripheral end of the nerve was stimulated with faradic current of increasing voltage and the presence or absence of contraction of the muscles of the leg was noted.

Myanesin in doses of 300 mg. per kg. (i.e., about 50 per cent of the LD50) did not cause paralysis of the muscle to indirect stimulation. Larger doses such as 500 mg. per kg. (80 per cent of the LD50) made even the strongest stimulation of the nerve ineffective after 30 min., but did not significantly alter the threshold to direct stimulation. The disappearance of the response to indirect stimulation could be caused by a depression of neuromuscular transmission or by paralysis of the nerve. It was of interest to ascertain whether other local anaesthetics, such as cocaine or procaine, could paralyse motor nerves when administered in very large doses. Cocaine in doses as large as 100 mg. per kg. (LD75) did not abolish or impair the response to indirect stimulation. Similar results were obtained with procaine. It does not appear possible to produce paralysis of peripheral nerves by the systemic administration of local anaesthetics such as cocaine or procaine. It is therefore unlikely that paralysis to indirect stimulation produced by large doses of myanesin could be due to a paralysing action on the nerve, particularly as myanesin is a less potent local anaesthetic than cocaine. Myanesin may have an effect on the nerve because of a selective affinity of the

nerve tissue for the drug, but no evidence in favour of this assumption is available. It appears more likely that myanesin can block neuromuscular transmission when administered in very large doses. This curare-like effect does not play any part in the production of reversible muscular paralysis with smaller doses because under such conditions paralysis to indirect stimulation was never observed.

The myanesin–strychnine antagonism

It has been shown previously that myanesin can antagonize the actions of strychnine (Berger and Bradley, 1946). In those experiments the drugs were administered subcutaneously in 2.5 per cent gum acacia solution. To measure the antagonistic effect more accurately a series of experiments was carried out in which the drugs were administered intravenously. With this mode of administration, variations due to differences in the speed of absorption were eliminated.

White mice weighing 18 to 22 grams were used. Injections were made into the tail vein at a rate of 0.3 c.c. per minute. The convulsant and myanesin were injected together in a volume of 0.4 c.c. per 20 grams body weight.

The minimal lethal doses (MLD), approximately equal to the LD80, of strychnine sulphate and leptazol were 0.43 and 100 mg. per kg. respectively. The median lethal dose (LD50) of myanesin was 320 mg. per kg.

Mice were injected with single minimal lethal doses of strychnine or leptazol, or multiples thereof. Myanesin was administered simultaneously and, for each dose of the convulsant, doses of myanesin were found which protected some of the animals from death. From these values the dose of myanesin protecting 50 per cent of animals was found graphically by plotting the probits of the percentage mortality against the log doses.

TABLE III

STRYCHNINE–MYANESIN ANTAGONISM AFTER SIMULTANEOUS INTRAVENOUS ADMINISTRATION TO MICE

Strychnine dose as multiple of MLD	Myanesin		Ratio to number injected of number		Dose of myanesin protecting 50% of mice mg./kg.
	mg./kg.	fraction of LD50	convulsed	died	
1	—	—	35/35	30/35	12
1	40	1/8	0/10	0/10	
1	20	1/16	2/10	2/10	
1	10	1/32	9/10	6/10	
2	80	1/4	0/20	0/20	34
2	40	1/8	17/20	7/20	
2	20	1/16	10/10	9/10	
3	320	1	0/10	0/10	80
3	160	1/2	6/10	2/10	
3	80	1/4	10/10	5/10	
4	320	1	7/20	12/20	160
4	160	1/2	20/20	10/20	
4	80	1/4	10/10	10/10	

The antagonistic action of myanesin against leptazol was relatively weak. About one quarter of the LD50 of myanesin protected 50 per cent of the animals against 1 MLD of leptazol. The effect of myanesin in maintaining life was greater than its power to prevent the occurrence of convulsions. Protection against 2 MLD of leptazol could not be obtained even when administered together with one LD50 dose of myanesin.

The antagonistic action of myanesin to the effects of strychnine was well marked. Animals could be protected from the effects of one MLD dose of strychnine by as little as one thirtieth of the LD50 of myanesin, and proportionally larger doses were able to antagonize larger doses of strychnine (Table III).

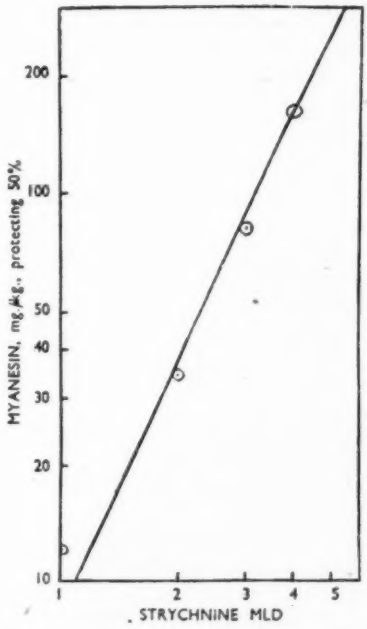

FIG. 1.—Antagonism of myanesin and strychnine. Abscissae: Dose of strychnine as multiple of MLD. Ordinates: Dose of myanesin protecting 50 per cent of animals.

When the median protective dose of myanesin was plotted against the dose of strychnine, expressed in terms of MLD, both values being plotted on a logarithmic scale, an approximately straight line was obtained (Fig. 1).

In suitable dosage myanesin antagonized all the effects of strychnine, and animals to which balanced mixtures of the two drugs had been administered appeared quite normal. The myanesin–strychnine antagonism was more complete than the hexobarbitone–strychnine antagonism ; in the latter it was not possible to find a balanced mixture, because doses of hexobarbitone which protected mice from convulsions and death caused a depression of greater duration and intensity in the presence of strychnine than in its absence.

Strychnine causes an increase of the reflex irritability of the spinal cord which may result in simultaneous contraction of all skeletal muscles if sufficiently large doses are given. Myanesin selectively antagonized the effects of strychnine in doses which by themselves did not produce any effects. It may therefore be inferred that myanesin decreased reflex hyperexcitability and prevented the passage of abnormal excitatory impulses through the reflex arcs.

Effect on tetanus

The effect of myanesin on experimental tetanus was examined in white mice. Ten micrograms of crude tetanus toxin were injected into the neighbourhood of the sciatic nerve high in the thigh. Local tetanus was apparent about 12 hours later and all animals died of generalized tetanus in about 3 days. Myanesin was administered intraperitoneally or subcutaneously at various times after tetanic spasms became apparent. In doses of

150 to 200 mg. per kg. it completely abolished the spasm in all stages of the disease. The board-like rigidity of the extremities gave place to complete flaccidity a few minutes after injection of the drug. Myanesin in these doses did not cause depression of respiration or any other untoward effects and poisoned animals under the influence of the drug were indistinguishable from control animals to which myanesin only had been given. The effect of the drug lasted for about 30 min. and was followed by a gradual reappearance of tetanus. Repeated doses of myanesin caused similar effects and regularly released the spasm. The impression was gained that the life of the animal could have been saved by continuous or frequently repeated administration of the drug.

The action of tetanus toxin on the central nervous system is similar to that of strychnine. The fact that myanesin can re-establish reciprocal innervation in conditions produced by these two different agents gives rise to the hope that it may also influence pathological excitatory innervation caused by degenerative processes of the central nervous system.

Effect on knee jerk

The effect of myanesin on the knee jerk was studied in cats. The animals were anaesthetized with chloralose intravenously and arranged for recording of the knee jerk as described by Schweitzer and Wright (1937). The limb was allowed to hang freely in order to facilitate observation of alteration in muscle tone. The jerk as a rule was elicited every 10 seconds.

Myanesin did not abolish the knee jerk of healthy cats. Intravenous doses ranging from 20 to 150 mg. per cat either did not cause any alteration or somewhat reduced the height of the myographic record (Fig. 2); the flexion at the knee joint was usually increased,

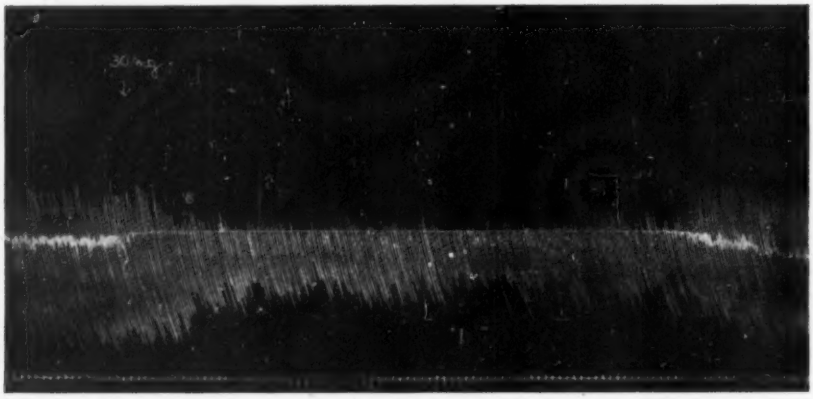

FIG. 2.—Cat 2.8 kg. Chloralose (0.08 g. per kg.). Record of knee jerk. Time intervals 30 seconds. At arrow 30 mg. myanesin slowly injected intravenously.

signifying a decrease in the tonus in the muscles, but complete inhibition of the jerk was not observed.

Some of the cats used in the experiments showed a very lively reflex followed by clonus. Exaggerated reflex excitability and tremors were also observed in certain animals under light chloralose anaesthesia (0.05 g. chloralose per kg.). Myanesin had a definite

effect on the knee jerk of these animals (Fig. 3A and B and Fig. 4). It inhibited clonus and tremors, abolished the irregular responses to patellar stimulation and reduced the knee jerk to its usual size. These effects could be produced with small doses of myanesin (20 to 30 mg. per 3 kg. cat). The effect set in almost immediately and lasted for about 20 minutes and sometimes longer.

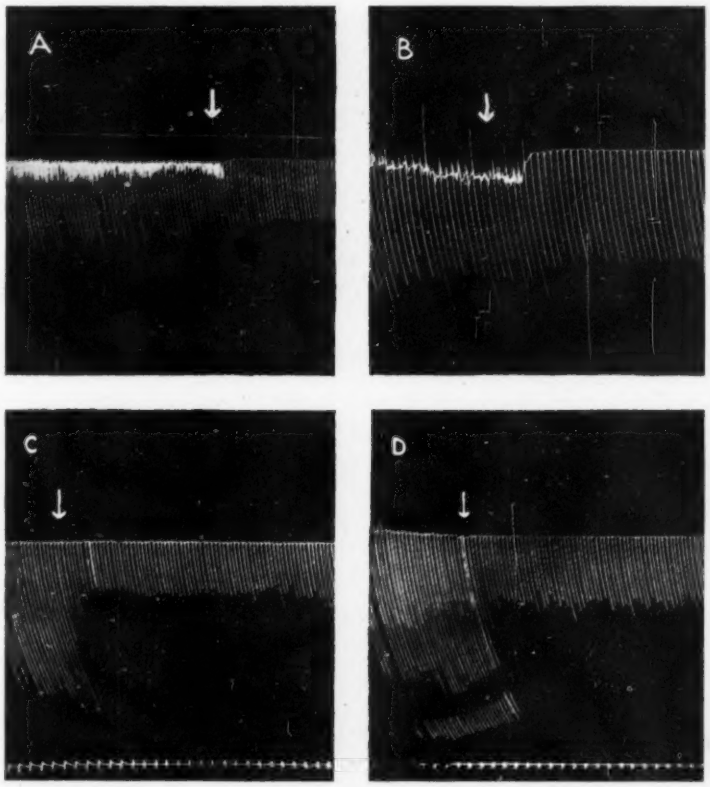

FIG. 3.—Records of knee jerk in cats weighing 2.8–3.1 kg. Jerk elicited every 10 seconds. Drugs injected intravenously. A. Chloralose 0.05 g. per kg., myanesin 50 mg. B. Chloralose 0.06 g. per kg., myanesin 150 mg. C. Chloralose 0.06 g. per kg., strychnine 0.2 mg., myanesin 50 mg. D. Chloralose 0.05 g. per kg., strychnine 0.4 mg., myanesin 100 mg.

The effect of myanesin on the experimentally increased knee jerk was also studied. Cats were injected with doses of strychnine insufficient to cause convulsions but producing exaggerated reflex activity. Myanesin in small doses promptly counteracted this increased reflex excitability and caused an immediate reduction of the reflex to the level present before administration of strychnine. (Fig. 3C and D.)

The effects of myanesin on the knee jerk of rabbits anaesthetized with urethane were also examined; they were similar to those observed in cats. Myanesin, 100 mg. per rabbit weighing about 3 kg., caused a decrease in the size of a few jerks immediately following the injection. Smaller doses did not influence the size of the jerk but abolished tremors, clonus and spontaneous movements of the leg.

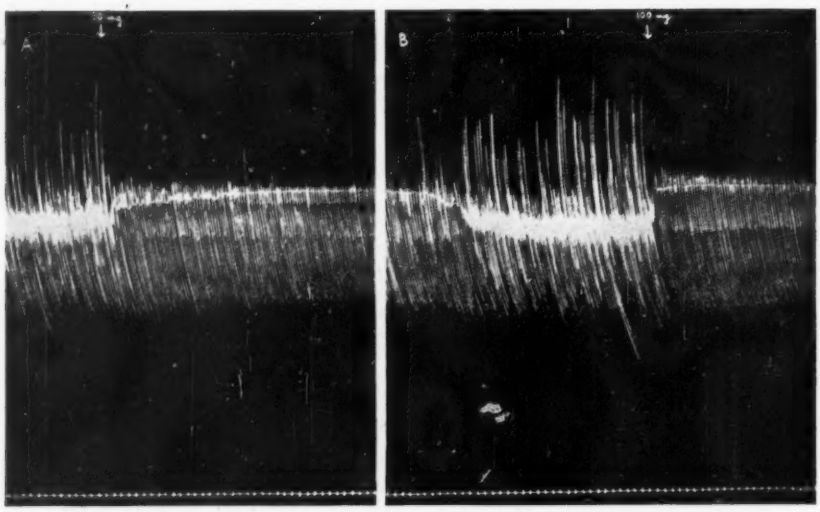

FIG. 4.—Record of knee jerk. Cat 3.4 kg. Chloralose 0.08 g. per kg. Time interval 30 seconds. At first arrow 30 mg. myanesin, at second arrow 100 mg. myanesin i.v. Between A and B a piece of the record occupying 40 minutes was cut out.

The experiments show that although myanesin has little effect on the normal knee jerk of the cat, it is very effective in reducing an exaggerated reflex to its normal size. Myanesin in suitable doses may therefore exert an inhibiting action on pathologically exaggerated functions of the central nervous system without influencing normal reflexes.

DISCUSSION

The experiments reported in this paper show that the effects produced by myanesin are due to its peculiar action on the spinal cord. Myanesin selectively depresses hyperexcitability of spinal reflexes, but hardly influences the normal reflex actions mediated through the cord. Symptoms of hyperactivity, whether due to the action of poisons or to light anaesthesia, can be inhibited with smaller doses of myanesin than those required for the depression of normal physiological functions. Strychnine convulsions in mice can be counteracted with as little as 20 mg. per kg. intravenously, but 150 mg. per kg. or more, administered by the same route, are required to paralyse the animal. In cats 20 mg. abolished tremors and clonus, but 150 mg. did not abolish the knee jerk. The relation between the dose required to bring hyperfunction back to normal and that causing depression of normal function was approximately the same with mice, rabbits, and cats.

The depressant action of myanesin on the peripheral nerves (the local anaesthetic action) is too weak to play any part in the effects produced by systemic administration of myanesin. In concentrations which can be achieved after systemic administration, myanesin does not exert any direct relaxing action on

muscles and does not block the action of acetylcholine on them. The curare-like action obtained with large doses is of toxicological interest only and never becomes apparent with doses from which animals recover. It is incorrect to call myanesin a curarizing agent, because doses causing relaxation during anaesthesia do not influence neuromuscular transmission, but produce relaxation by a depressant action in the spinal cord.

Muscular relaxation produced by myanesin differs from that produced by curare not only in that the drugs act on different structures, but also in the order and degree in which various groups of muscles are affected. With curare, muscles with cranial innervation are affected first ; the peripheral and intercostal muscles are paralysed next, and with complete curarization the diaphragm is affected to almost the same extent as other muscles. With myanesin the muscles of the posterior half of the body are affected first, next the peripheral and intercostal muscles, then the cranial muscles ; the diaphragm is affected last and there is a distinct margin between doses causing muscular paralysis and those causing arrest of respiration.

Both curare and myanesin have been used for the production of muscular relaxation during anaesthesia (Mallinson, 1947). Curare is the agent of choice for complete suppression of respiration, such as is required during certain operations on the lungs. Myanesin, on the other hand, appears to be more useful for the production of muscular relaxation when suppression of respiration is not desired. Myanesin should never be used for the production of respiratory arrest, because the doses required for this purpose are large and affect the heart and blood pressure.

Occasionally when an attempt was made to produce respiratory arrest in an already paralysed rabbit by rapid intravenous injection of myanesin, a general rigidity without arrest of respiration developed. The rigidity, which in appearance was similar to decerebrate rigidity, lasted for about 1 min. and was followed by profound muscular relaxation from which the animals recovered. The cause of this symptom is not understood. It may be due to a direct action on the muscle. It was observed only in certain rabbits after rapid intravenous injection of concentrated solutions. It was not seen in anaesthetized animals. Other species of animals, as well as most rabbits, showed respiratory arrest if administration was continued after complete paralysis was obtained.

The rigidity observed after rapid intravenous injection to certain non-anaesthetized rabbits is the only sign of stimulation which has been observed with myanesin. With this exception myanesin caused pure depression in all species which were examined and at all dose levels. Crystalline *d*-tubocurarine chloride, on the other hand, may cause conspicuous signs of central stimulation such as trembling, hyperexcitability to stimuli, muscle twitching, and convulsions. These symptoms are particularly well marked in mice and rats, but can also be observed in other species (Cohnberg, 1946).

West (1935) observed that certain samples of curare removed the violent spasm of parathyroid tetany in dogs without paralysing the animal. This selective removal of pathological rigidities without apparent diminution of voluntary power was termed lissive action by West. Pure crystalline tubocurarine chloride possessed no trace of lissive power in dogs.

Experiments reported in this paper indicate that myanesin possesses a lissive action. It should, therefore, prove useful in the treatment of spastic paralysis, hypertonic states, and tremors. It may be expected that myanesin will be effective in these conditions in doses which do not affect consciousness, do not diminish muscular power, and do not cause side effects. Similar results may also be expected after oral administration because myanesin is well absorbed from the intestinal tract.

Summary

1. Myanesin in high dilution had no direct action on the rectus abdominis of the frog and did not block the action of acetylcholine on this muscle.

2. The local action of myanesin on peripheral nerves was similar to that of procaine.

3. In doses causing reversible paralysis, myanesin did not possess a curare-like action, but toxic doses had a blocking effect on the myoneural junction.

4. Myanesin had but little effect on the normal knee jerk. An exaggerated knee jerk due to light anaesthesia, strychnine, or unknown causes was promptly reduced to the usual size.

5. Myanesin in small doses antagonized all the effects of strychnine and relieved tetanic spasm.

6. Myanesin had a selective depressant action on the spinal cord. In doses which had little effect on voluntary power it restored deranged reciprocal innervation to normal and counteracted symptoms caused by a release from inhibitions as observed during light anaesthesia. The powerful effect of myanesin on tremors, increased reflex excitability, and similar symptoms suggests that it may be useful in the treatment of spastic and hypertonic conditions.

I wish to express my thanks to the Directors of The British Drug Houses, Ltd., for their interest in the work and their permission to publish the results. My thanks are also due to Mr. R. A. Hall and to Misses B. J. O'Brien, L. F. Carrick, D. M. Culver, D. M. Gurney, B. Hall, E. U. Haywards and M. E. L. Tattersall for technical assistance.

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THE USE OF DRUG ANTAGONISTS FOR THE IDENTIFICATION AND CLASSIFICATION OF DRUGS

BY

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A sensitive biological method for the identification of drugs has been described by Chang and Gaddum (1933). It consists in estimating the activity of an unknown substance in terms of a standard, quantitatively, in several different pharmacological tests. As a rule the ratio of activity between standard and unknown in different tests is only constant if the two samples are chemically identical, otherwise the ratio of activity in different tests varies. By this method even closely related substances may be differentiated.

Another biological method for the identification and differentiation of drugs consists in testing quantitatively their responses towards antagonists. It has been shown in a previous communication (Schild, 1947) that the effects of histamine and acetylcholine may be thus distinguished, since many antagonists although unspecific at high concentrations will differentiate between these drugs at lower concentrations. The present investigation is concerned with the question whether drugs which are more closely related than histamine and acetylcholine may also be differentiated in this way, or whether antagonists, even though they may be used in an exact and quantitative manner, would fail to discriminate between closely related substances.

If indeed some drugs, although distinguishable chemically or by their relative activity in different pharmacological tests, could not be distinguished by their reactions to antagonists, they might conveniently be grouped into a common pharmacological class. It will be shown that drugs can in fact be found which are related in this way, and that if a certain simple scheme of drug action be accepted a rational classification of active drugs based on their responses to drug antagonists may be attempted.

METHODS

The experiments were done on the isolated ileum of the guinea-pig, employing the apparatus for assaying antagonists on isolated tissues which has previously been described (Schild, 1947).

Plan of experiments.—The object of these experiments was to find out whether drugs could be differentiated by their responses to antagonists. They were accordingly designed to test whether the effects of two given stimulant drugs would be reduced by some antagonist to the same extent or not.

The assays are performed in two stages. In a preliminary experiment doses of the two active drugs producing approximately equal submaximal effects are determined as well as a concentration of the antagonistic drug sufficient to reduce these effects without completely abolishing them. In a final experiment these drugs are administered in the presence and in the absence of the antagonist in a planned sequence, which must be statistically unbiased in order to allow a statistical analysis of the results of each experiment to be made.

A typical experiment is shown in Fig. 1. The order of addition of drugs to the bath is as follows. The doses are administered in sets of four, each set consisting of a random sequence of one dose of each of the active drugs alone and a further dose of each of the active drugs in the presence of the antagonistic drug (after a preliminary period of contact between tissue and antagonist of 2 min.). After each administration of the antagonist several doses of the active drug alone are interjected in order to let the tissue recover, if possible, from the preceding depression.

Persistent depression produced by antagonists is the most serious difficulty in planning an unbiased sequence of doses suitable for statistical analysis. The depressant effect is partly overcome by interposing doses of the active drug alone. More fundamentally, the arrangement of the experiment itself is such that each of the active drugs has an equal chance of being preceded by a depressant injection, and it is thus unbiased with regard to the main point at issue. It is merely a matter of convenience how many " recovery " periods of active drug alone are interposed and it may in some cases be more advantageous to have a fixed small number of such periods rather than to wait until the response has reached a steady state.

A complete experiment consists of several sets (randomized groups) of four doses. The final effect is assessed by averaging the effects obtained in the individual sets. If the two drugs are depressed to the same extent by the antagonist, and $\bar{y}_A$ and $\bar{y}_B$ are the mean effects produced by drugs A and B, and $\bar{y}_{AZ}$ and $\bar{y}_{BZ}$ the mean effects produced by these drugs in the presence of the antagonist Z,

$$\bar{y}_A - \bar{y}_{AZ} = \bar{y}_B - \bar{y}_{BZ}$$

or

$$\bar{y}_A + \bar{y}_{BZ} = \bar{y}_B + \bar{y}_{AZ}$$

The latter equation is tested statistically by an analysis of variance carried out as previously described (Schild, 1942). In practice there is usually some deviation from theoretical equality, but unless this is statistically significant it may be assumed that both drugs have been depressed to the same extent.

Use of a Drug Antagonist for the Identification of an Unknown Substance

Curare causes the release of a histamine-like substance from the perfused gastrocnemius muscle of the dog (Alam *et al.*, 1939). In the present experiment a dog's hind limb was perfused with defibrinated blood by means of a Dale-Schuster pump, the blood being oxygenated through the animal's own lungs ; a solution of 50 mg. crude curare was injected into the artery perfusing the limb and the effluent was collected. The blood was extracted by Code's method and the extract assayed on the guinea-pig's ileum. The apparent concentration

of histamine in the sample was 500 μg. per l. as against 16 μg. per l. in the controls.

In order to identify the substance further the effect of a low concentration of benadryl (dimethylaminoethyl benzhydryl ether hydrochloride) on it was compared with the effect of the same concentration of benadryl on histamine. The experiment is illustrated in Fig. 1. A concentration of benadryl of 1 : 150

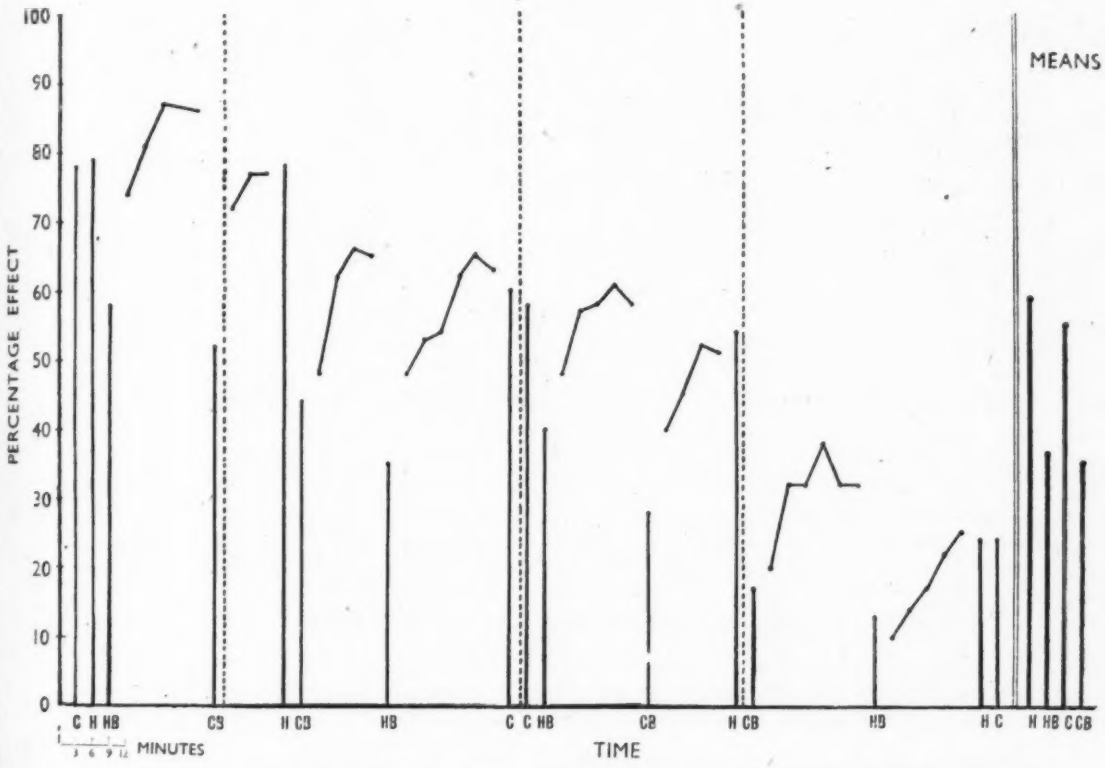


FIG. 1.—Assay to test whether the effects of histamine (H) and an unknown substance (C) released from skeletal muscle by curare are reduced to the same extent by benadryl (B). Sequence of injections explained in the text. Vertical lines represent the effects of test doses of the two drugs given in the presence and in the absence of the antagonist, each effect being repeated 4 times. The irregular curves represent the effects of a constant dose of histamine given in order to allow the preparation to recover from a preceding dose of benadryl. Note incomplete reversal of depression.

million produced an appreciable reduction of the effects of both histamine and the unknown substance. There also occurred a progressive deterioration of the preparation, presumably due to the cumulative effects of the antagonist. In spite of this and the unequal effects produced by individual doses of the antagonist, the mean reduction of effect of the two active drugs is almost identical.

The results of an analysis of variance of this experiment are shown in Table I. In the last column the mean square for each item should be compared with the mean square for

error. Two of the mean squares are smaller than the error mean square and thus statistically obviously not significant. One of these is the mean square which assesses the relative reduction of the effects of histamine and the extract by benadryl. Since there is no signifi-

TABLE I

ANALYSIS OF VARIANCE OF BENADRYL DEPRESSION OF HISTAMINE AND HISTAMINE-LIKE SUBSTANCE RELEASED BY CURARE

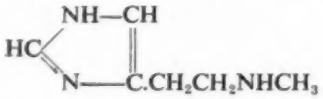
Source of variation	Sum of squares	Degrees of freedom	Mean square
Between successive "randomized groups"	4805	3	1602
Between histamine and extract	25	1	25
Between active drugs alone and active drugs + benadryl ("slope")	1764	1	1764
Between depression of histamine effect by benadryl and depression of extract effect by benadryl	6	1	6
Error	502	9	56
Total	7102	15	

cant difference the reduction of the two effects may be assumed to be equal and the main object of the experiment is achieved. A further incidental result is a lack of statistically significant difference between the effects produced by histamine and the extract, indicating that the activity of the extract might reasonably be assumed to be 500 μg. per l. as originally assumed. Two of the mean squares are much higher than the mean square for error and statistically significant. One of these is the mean square for "slope" indicating that the concentration of benadryl used produced a significant reduction of effect. The other is the mean square between successive "randomized groups," showing that a real change in sensitivity, in this case a progressive deterioration, occurred in the course of the experiment.

Similar results were obtained in two further experiments.

It was concluded that since benadryl does not discriminate between the two substances they are likely to be related or possibly identical.

Drugs which are Indistinguishable by their Reaction to Antagonists

N-methylhistamine, 

has been synthetized by Garforth and Pyman (1935). Its pharmacological actions were investigated by Vartiainen (1935) who found it to be twice as active as histamine on the guinea-pig's ileum and about half as active as histamine on the cat's blood pressure. Since histamine and its N-methyl derivative are closely related, as well as clearly distinguishable by their relative activity in different pharmacological tests, they were selected as a representative pair to investigate the effect of antagonists on closely related substances.

In the following experiments the effects of three different antagonists on the action of equiactive concentrations of histamine and N-methylhistamine were

studied. The N-methyl derivative was approximately 2.5 times as active as histamine in terms of molar concentrations. The antagonists used were antergan (N:N-dimethyl-N′-phenyl-N′-benzylethylene diamine hydrochloride; Halpern, 1942), pethidine, and atropine. The results are illustrated in Fig. 2.

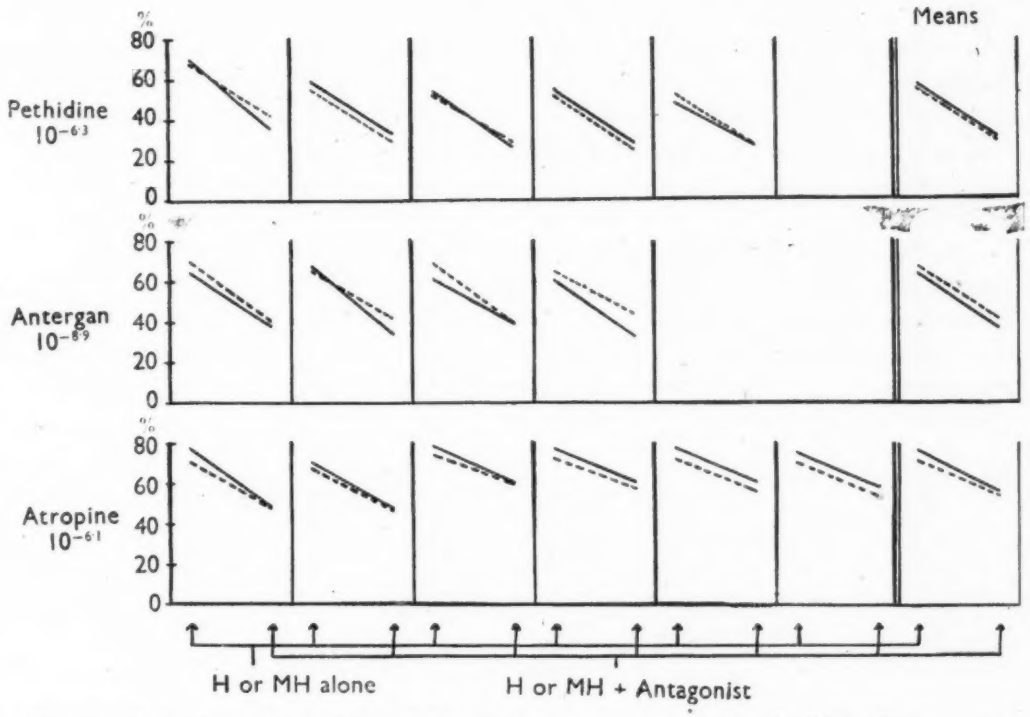


FIG. 2.—Reduction of the effects of histamine (– – – – –) and N-methylhistamine (———) by three different antagonists. The molar concentration of histamine used was approximately 2.5 times that of N-methylhistamine.

Points in the top left-hand corner of each square represent the effects of histamine or N-methylhistamine alone, the lower points the effect of these drugs in the presence of a constant concentration of antagonist. Successive squares in a horizontal row represent effects in successive "randomized groups" and the final square the arithmetical mean of effects, all obtained on the same strip of intestine.

The results were analysed statistically as in the previous example and it could be shown that a given concentration of each antagonist produced, within statistical limits, the same reduction of effect of the two stimulant drugs. It was concluded that none of the three antagonists is capable of discriminating between histamine and N-methylhistamine.

These experiments show that certain drugs react in an analogous manner towards antagonists of very different chemical and pharmacological nature. It is doubtful if any antagonist could be found to discriminate between histamine

and its N-methyl derivative. If the unknown substance discussed in the preceding section had in fact been N-methylhistamine it could not have been distinguished from histamine by means of these antagonists.

Use of Antagonists for the Classification of Drugs

It might reasonably be assumed that drugs which in a given pharmacological test could not be differentiated by their reaction towards antagonists, or, more precisely, drugs which in the presence of any effective antagonist showed equal reductions of equal effects, would be closely related in their pharmacological action on the particular test object used. Conversely, drugs which could be differentiated by their reactions towards most antagonists except the most unspecific ones might be said to be pharmacologically unrelated.

In thus using the effects of antagonistic drugs as a criterion for classifying active drugs, the following scheme of drug action might perhaps be visualized. It may be assumed that between the first impact of a drug on the tissue and its final effect, muscular contraction in this case, a series of successive processes occur with any of which antagonists can interfere. Two successive stages are indicated in Fig. 3.

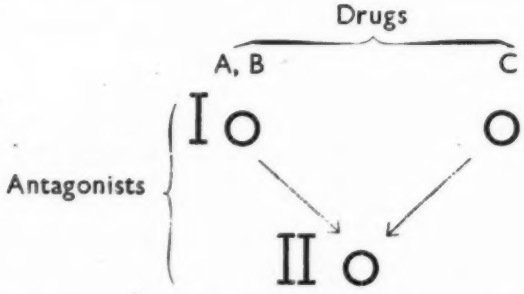


Fig. 3.—Site of action of antagonists. If A and B stand for histamine and N-methylhistamine and C for acetylcholine, it is possible that small concentrations of antagonists may act at one of the primary sites only, whilst larger concentrations may also antagonize a later common reaction.

If two drugs have different primary points of impact their reactions must sooner or later converge into a final common path. An antagonist may interfere with the action of a stimulant drug either before or after a common final path with some other drug is reached. Thus antagonist I reduces only the action of drugs A and B, but not that of C, whilst antagonist II reduces A, B and C. It may further be assumed, at least as a first approximation, that an antagonist acting at a given site would reduce the effect of those drugs with whose pathways it interferes to the same extent. Thus antagonist I would depress A and B equally and antagonist II A and B and possibly C equally.

It follows that if two drugs such as A and B act by the same mechanism, their effects will be reduced to the same extent by antagonists, independently of whether the latter exert their action by competing for a primary site or by

interfering with some other reaction involved in the contractile process. Such drugs might well be classed together into a primary pharmacological class.

Antagonists may of course act at more than one site. A possible explanation for the dual action of drugs such as atropine and neo-antergan (Schild, 1947), which antagonize both histamine and acetylcholine at relatively high concentrations, but only one of the two at low concentrations, would be that at low concentrations the antagonists act at the level of I only and at high concentrations at the level of II as well as of I. Another way of explaining the dual action would be to assume that at high concentrations neo-antergan begins to affect the primary site for acetylcholine and atropine to affect the primary site for histamine. At any rate it is clear that at higher concentrations these antagonists must act on at least two sites.

The scheme is capable of expansion to include the action of further groups of drugs and their antagonists. A scheme of this kind cannot, however, account for special types of antagonism, such as a chemical combination between active drug and antagonist.

Discussion

One of the earliest attempts at classifying drugs by their reaction to antagonists was the classification of substances contracting plain muscle into musculotropic and neurotropic according to their reaction to atropine. Various objections have been raised from time to time to this conception.

It was pointed out by Magnus (1905) that using the criterion of atropine to localize the site of action of drugs in the intestine implied firstly that atropine had only one site of action in the wall of the intestine and secondly that the effect of atropine could not be reversed by some other drug acting on the same site. Both these assumptions were unlikely to be true. It was indeed shown by Magnus himself that atropine had more than one site of action in the intestine and that it would antagonize at different concentrations drugs belonging to quite different groups such as pilocarpine and barium, and it had been shown by Langley and others that there existed a quantitative antagonism between atropine and pilocarpine. This line of criticism does not necessarily invalidate the use of drug antagonists for localizing the site of action of drugs; rather it points to the necessity of refining these methods by using drug antagonists in a more quantitative way. In practice the setting apart of a group of "muscarinic" drugs (Dale, 1914), exceptionally sensitive to atropine, has been extremely fruitful, and although the localization implied in the term "neurotropic" cannot be maintained any longer, these drugs must still be regarded as forming a group apart, likely to have a common mechanism of action.

From another point of view the old classification has been criticized by Winder *et al.* (1946). These workers came to the conclusion that a subdivision into two groups was inadequate, since in addition to the acetylcholine group, a histamine and a barium group of plain muscle stimulants could be clearly dis-

tinguished by their differential reactions towards antagonists. There is, however, no special reason for confining plain muscle stimulant drugs to three types only and it would seem reasonable to look for further types to be differentiated by their reactions towards antagonists. This is precisely what the present classification proposes to do, since drugs which react quantitatively alike to antagonists are placed together, and drugs which can be differentiated by their quantitative response to antagonists are separated.

The first distinguishing feature of the proposed classification is that it relies upon a quantitative discrimination, the second that it relies upon the response to several antagonists rather than to a single one ; only those drugs are assigned to a primary pharmacological class (in relation to a given tissue) which respond in a quantitatively identical manner to every effective antagonist. This method of classification has the merit of being sharply defined, but it may ultimately prove to have been too rigidly conceived. In a more general way, however, quantitative similarity in behaviour to antagonists is bound to denote some pharmacological relationship between drugs, and the recognition of these relationships may eventually lead to a better understanding of the mechanism of action of drugs.

Summary

1. A method is described for evaluating statistically whether the effects of two drugs are reduced equally by antagonists.

2. If equal effects are produced on the guinea-pig's ileum by histamine and N-methylhistamine, they are antagonized to the same extent, quantitatively, by effective concentrations of three different antagonists. Similarly the effects of histamine and a histamine-like substance released from striated muscle by curare are equally depressed by an antagonistic drug. It is concluded that antagonists probably cannot be used to discriminate between closely related drugs.

3. A scheme of drug action is proposed which can serve as a basis for a classification of active drugs by means of drug antagonists.

I am indebted to Dr. A. L. Morrison of the Roche Research Department for preparing N : N-dimethyl-N′-phenyl-N′-benzylethylene-diamine, and to Dr. Harold King for a supply of N-methylhistamine.

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THE INFLUENCE OF CALCIUM AND POTASSIUM IONS ON THE TOXICITY OF OUABAIN

BY

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In the mass of literature dealing with digitalis and Ca and K ions there are no quantitative results showing the influence of varying amounts of these ions on the action of a constant dose of glycoside ; nor are there any laboratory observations on the effects of Ca or K on the symptom of digitalis vomiting. Accordingly the following studies have been made.

Anaesthetized rabbits have been used to determine (i) the lethal dose of ouabain infused intravenously at constant rate in physiological saline, (ii) the lethal dose of the same concentration of ouabain infused together with increasing concentrations of $CaCl_2$, and (iii) the lethal dose of the same concentration of ouabain infused together with increasing concentrations of KCl.

Perfused rabbit hearts have been used to determine the effect of alterations in the amount of (a) $CaCl_2$ and (b) KCl in the Locke solution on the effect of a fixed concentration of ouabain.

Pigeons have been used to determine whether the injection of KCl modified the action of ouabain in causing vomiting.

Experimental Results

(*a*) *Anaesthetized rabbits*

Method.—Thirty-four animals were used. All were given urethane by ear vein till full surgical anaesthesia was reached, the dose necessary being remarkably constant at 1.55 g. (6.2 ml. of 25 per cent (w/v) in distilled water) per kg. of rabbit given over 10–15 min. Urethane was used in order to avoid ether, which increases the scatter of results of digitalis assay by the cat method (Burn, 1937) ; the quantity agrees well with the 1.8 g./kg. usually recommended for cats intramuscularly. Cannulae were inserted into the trachea, the left carotid artery for recording the blood pressure by mercury manometer, and the left femoral vein for the infusion of solutions. Artificial respiration was given by a pump as soon as an animal's respiration began to be shallow. Into all animals, except controls, ouabain (20 μg./ml.) was infused in physiological saline at the constant rate of 0.15 ml./min./kg. rabbit. This rate of infusion is within the optimum range (Rapson and Underhill, 1935). Observations on the effects of Ca and K on the lethal dose of ouabain were made by adding varying amounts of $CaCl_2$ or KCl to the infusion, and the end-point of an infusion was taken as the point at which blood pressure suddenly fell to zero without recovery. The animals were thus in the following groups:

(i) Receiving ouabain alone.
(ii) Receiving ouabain + 1, 2 or 3 per cent (w/v) $CaCl_2$.

(iii) Receiving ouabain + 0.5, 1 or 2 per cent (w/v) KCl.
(iv) Controls receiving 3 per cent $CaCl_2$ alone.

The control animals needed more than 0.5 g. $CaCl_2$/kg. to kill them, in agreement with Nahum and Hoff (1937) who gave 10 per cent $CaCl_2$ at 2 ml./min. In the present series none of the rabbits receiving ouabain and calcium received more than 0.09 g. $CaCl_2$/kg. Since the effects of KCl on digitalis toxicity were protective, no controls with KCl alone were considered necessary.

Results.—In order to compare the relative effects of $CaCl_2$ and KCl on the action of ouabain, concentrations were expressed as molarities, but percentage concentrations (w/v) were also recorded for convenience (Table I). The mean results are represented graphically in Fig. 1 in order to show the difference

TABLE I

RABBITS. Femoral vein infusions of 20 μg. ouabain/ml. at 0.15 ml./kg./min.

Fluid infused		Individual LD ouabain μg./kg. rabbit	Mean
Ouabain and $CaCl_2$ Amount of $CaCl_2$			
Molarity × 10	Per cent		
2.7	3	30.5, 36.5, 35.7, 58.0, 58.0	43.7
1.8	2	60.0, 51.3, 73.5, 49.6, 74.7	61.8
0.9	1	75.5, 105, 93.3, 65.8, 56.0	79.1
Ouabain alone		114, 77.7, 81.0, 117, 88.0	95.5
Ouabain and KCl Amount of KCl			
Molarity × 10	Per cent		
0.67	0.5	148, 105, 114, 123	123
1.3	1	121, 145, 132, 166, 141	141
2.7	2	171, 151, 156, 130	152

in slope of the curves relating mean LD ouabain (ordinates) to concentration of $CaCl_2$ or of KCl (abscissae) in the infusion. Clearly there is a qualitative difference in effect, increased $CaCl_2$ causing a linear decrease in LD ouabain, while increase of KCl causes an increase in LD ouabain ; there also appears to be a quantitative difference, because the curve for KCl is the steeper. With the two lowest concentrations of potassium used, unit change of concentration had a greater effect on the LD of ouabain than had unit change in concentration of $CaCl_2$; thus, 1, 2, and 3 per cent $CaCl_2$ decreased the LD ouabain by 17, 36, and 54 per cent respectively, whereas 0.5, 1, and 2 per cent KCl increased it by 29, 47, and 59 per cent. This suggests that the action of ouabain is influenced more by the absolute concentration of potassium in a perfusion fluid than by the potassium/calcium ratio, at least over a certain range. In order to

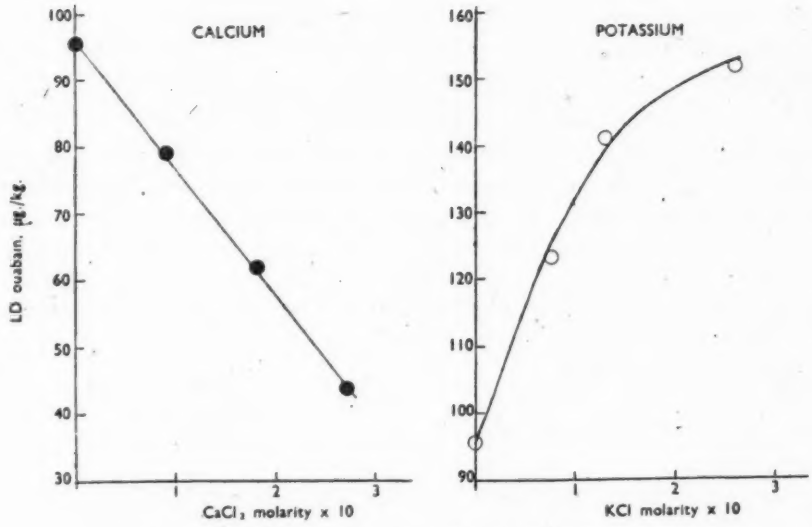

FIG. 1.—Rabbits. Femoral vein infusion of ouabain (20 µg./ml.) at a rate of 0.15 ml./kg./min. The relation between the LD ouabain and the Ca or K content of the infusion.

test this point it was necessary to perform experiments in which potassium or calcium concentrations could be lowered as well as raised, and accordingly the isolated perfused rabbit heart was used.

(*b*) *Perfused rabbit hearts*

Method.—Thirty-six animals were used. Animals were killed by a blow on the head and bled out by cutting the throat. The heart was rapidly cut out, dissected clean and perfused with oxygenated Locke's solution (percentage composition (w/v) as follows: 0.9 NaCl, 0.042 KCl, 0.024 $CaCl_2$, 0.1 dextrose, 0.05 $NaHCO_3$) at 36° by the Martin-Langendorff method. A hook was passed through the apex of the ventricles and the amplitude of beat recorded on a smoked drum by a lever and writing point. Rate and amplitude of beat and coronary flow were allowed to become steady, and from 30–45 min. after setting up the preparation perfusion was commenced with ouabain (0.4 µg./ml.) in the Locke's solution. The measure of toxicity of the ouabain was taken as the time it took to reduce the amplitude of beat to 50 per cent of its original value. This time is referred to as the "survival time." Thus anything which increased the toxicity of the ouabain *shortened* the survival time, and anything which decreased the toxicity *lengthened* the survival time. All hearts except the controls received 0.4 µg. ouabain/ml., and were divided into the following groups:

(i) Receiving ouabain in normal Locke's solution.

(ii) Receiving ouabain in Locke with half or twice the normal $CaCl_2$.

(iii) Receiving ouabain in Locke with 0.5, 1.25, and 1.5 times the normal KCl.

(iv) Control hearts receiving no ouabain. With the highest and lowest concentrations of $CaCl_2$ or KCl used all hearts survived for long periods.

(v) Hearts, in normal Locke containing 0.4 µg. ouabain/ml., in which toxic effects were caused to disappear by changing to perfusion fluid containing excess KCl.

Results.—For the reasons already given, the concentrations of $CaCl_2$ and KCl were expressed in molarities. These results (Table II) are plotted graphically (Fig. 2) as mean survival times against molar concentrations of $CaCl_2$ or KCl, and, as for the anaesthetized rabbits, it will be seen that when potassium is increased above normal, unit change in concentration of potassium produces a bigger effect on survival time than unit change in calcium concentration when calcium is decreased. This is expressed more clearly (Fig. 3), when the same

TABLE II

RABBIT HEARTS perfused by Langendorff's method with 0.4 μg. ouabain/ml.

Concentration in Locke $CaCl_2$		KCl		Ratio	Individual survival times in minutes	Mean survival times in minutes
Molarity × 10^3	mg. per 100 c.c.	Molarity × 10^3	mg. per 100 c.c.	K/Ca		
2.16	24	5.63	42	2.61	32, 39, 49, 50, 51, 34	38
4.32	48	,,	,,	1.30	23, 28, 14, 40, 13	24
1.08	12	,,	,,	5.22	60, 39, 45, 28, 49	44
2.16	24	2.81	21	1.30	20, 13, 14, 16	16
,,	,,	7.03	52.5	3.26	92, 110, 45, 48, 66	72
,,	,,	8.44	63	3.91	87, 114, 195, 207, 254	171

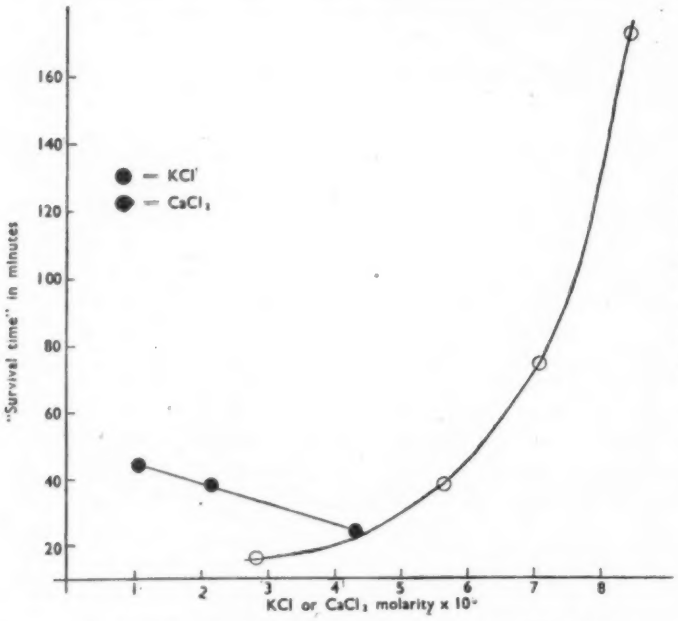

FIG. 2.—Langendorff rabbit hearts; 0.4 μg. ouabain/ml. Locke's solution at 36° C. The relation between the time required to reduce the amplitude of the beat to 50 per cent of its original value ("survival time") and the Ca or K content.

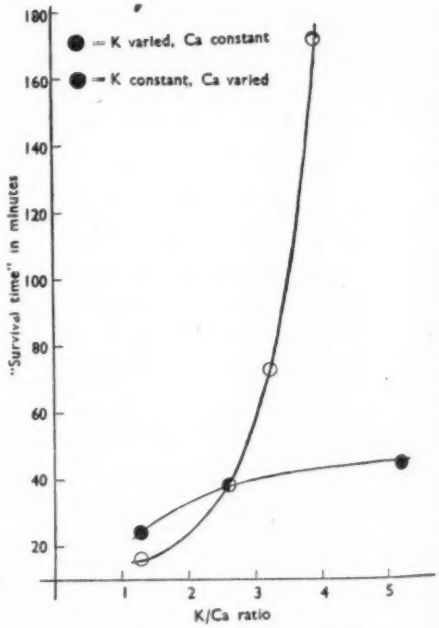

FIG. 3.—Same as Fig. 2. The relation between "survival time" and the K/Ca ratio.

survival times are plotted against the K/Ca ratio in the perfusion fluid. If the alteration in the ouabain effect had been solely a function of the K/Ca ratio, the same effect would have been obtained at a given K/Ca ratio whether it was attained by increasing K, or by decreasing Ca, but as Fig. 3 shows the absolute concentration of K had a relatively greater importance.

In addition to the quantitative results with potassium, three hearts were perfused with 0.4 μg. ouabain/ml. in normal Locke until gross irregularities of beat occurred, usually within 25 min.; potassium was then added to the perfusion fluid to increase its KCl content by 50 per cent. This abolished the irregular rhythms, caused systole to lessen, and prolonged the survival time, which would have been an hour at most, to several hours. A record of one of these hearts is shown in Fig. 4, and is in agreement with the results of Sampson *et al.* (1943) on the human being.

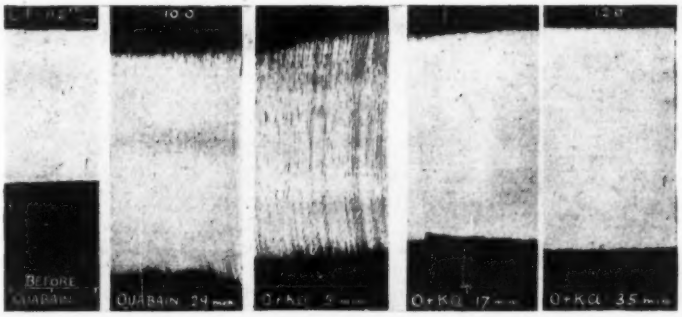

FIG. 4.—Langendorff rabbit heart. KCl abolition of irregular rhythm due to 0.4 μg. ouabain/ml. Tracing reads from left to right. Time marker in minutes. Between (i) and (ii) ouabain added to the Locke to contain 0.4 μg./ml. Between (ii) and (iii) KCl concentration raised by 50 per cent, with abolition of irregular beats (iv) and (v).

(*c*) *Pigeon emesis*

Apparently the effect of K on the vomiting produced by digitalis bodies has never been investigated, except for the observation of Sampson *et al.* (1943) that the nausea and vomiting of their patients who received an overdose of digitalis was not affected by potassium acetate administered orally. The pigeon-emesis method of digitalis assay introduced by Hanzlik and Schoemacher (1926), when modified as Burn (1930) suggested, affords a convenient method of testing this action of K against digitalis; if K were to lessen digitalis vomiting, this clearly would indicate antagonism of an extra-cardiac effect of digitalis, since digitalis vomiting has been shown to be independent of cardiac connections with the C.N.S. (Hanzlik and Wood, 1929; Haney and Lindgren, 1942).

Methods.—Twenty pigeons were injected with 15 μg. ouabain/300 g. pigeon into the wing vein on two separate occasions. The same pigeons were injected on two other occasions (interpolated between these two) with 15 μg. ouabain and 6 mg. KCl per 300 g. pigeon. This amount of KCl was found to be about the maximum which pigeons would tolerate, for in a trial with KCl alone it killed one bird in a group of 20. The number vomiting is recorded in Table III. When the pigeons were injected with ouabain alone 22 out of 40 injections caused vomiting; with ouabain and KCl, 19 out of 40 injections caused vomiting. Clearly potassium did not protect pigeons against the vomiting induced by the ouabain.

TABLE III

PIGEON EMESIS RECORD

Pigeon number	Ouabain 15 μg./300 g.		Ouabain 15 μg. and KCl 6 mg./300 g.	
	24.3.47	2.4.47	27.3.47	31.3.47
1	+	+	+	+
2	+	+	+	+
3	0	+	0	0
4	0	+	+	+
5	+	+	+	+
6	+	+	+	+
7	+	0	0	+
8	+	+	0	+
9	0	0	0	0
10	+	+	0	+
11	+	+	0	+
12	0	0	+	0
13	0	0	0	0
14	0	0	0	+
15	0	0	0	0
16	+	+	+	0
17	0	0	0	+
18	0	+	0	+
19	+	0	0	0
20	0	+	0	0
Total vomiting ..	10	12	7	12

Discussion

Although there was reliable information concerning the effects of cardiac glycosides on frog hearts by the middle of the nineteenth century (Vulpian, 1855), it was not until after Ringer's proof of the importance of certain ions in the perfusion fluid (Ringer, 1883) that calcium and potassium became suspected of playing a part in the action of these glycosides. In frog hearts either Ca excess or K deficiency increased the action of digitalis, while Ca deficiency or K excess lessened its effect (Werschinin, 1910; Clark, 1912; Konschegg, 1913). From these results it was concluded that digitalis antagonized K just as Ca did. Some extreme views emerged; while Burridge (1915–16) postulated that the cardiac glycosides act by sensitizing the heart to calcium, Weizsäcker (1917) drew a similar conclusion that digitalis improves the force of the heart only when there is a lack of calcium. Loewi (1918) reiterated Burridge's contention, and others wrote in support (Geiger and Jarisch, 1922; Grumach; Grünwald; Handovsky; Hoffmann; Schoen; 1923). Pietrkowski (1918), however, maintained that the effects of low Ca on the action of digitalis could be countered by increasing the sugar of the perfusion fluid, and concluded that digitalis has a direct action on the heart. All these observers were concerned with the systolic action of digitalis, and with the systolic effect of calcium, whereas it has been shown (Werschinin, 1910; Cushny, 1925) that small doses of digitalis result in

diastolic arrest. Recently this observation has also been brought into line with the theory that digitalis sensitizes the heart to calcium (Blumenfeld and Loewi, 1945).

In mammals the occurrence of calcium and digitalis synergism has been well established both in animal experiments (Lieberman, 1932 ; Schunterman, 1935 ; Gold and Kwit, 1937 ; Gold and Edwards, 1927 ; Bower and Mengle, 1936 ; Golden and Brams, 1938), and in the human being, Edens and Huber (1916) finding that patients prone to digitalis pulse bigeminy have a high blood calcium, while Bower and Mengle (1936) record two cases of sudden death after intravenous calcium salts following digitalis.

In contrast to the foregoing results are those of Fischer (1928) who found that digitalis sensitizes the heart to all stimuli, e.g., Ca, ethyl alcohol, or K, and of Camp (1939) who also found the heart after treatment with digitalis to be sensitized to K. Nahum and Hoff (1937), however, and Smith, Winkler, and Hoff (1939) failed to obtain Ca and digitalis synergism. Nyiri and DuBois (1930) disagree with the extreme view of Loewi, maintaining that digitalis can exert its full effect in the complete absence of calcium in the fluid perfusing a frog's heart, though they agree that excess of calcium enhances its action.

A new line of evidence that K is involved in digitalis action arises first from the work of Calhoun and Harrison (1931). They showed that toxic doses of digitalis lower the level of cardiac K ; the effect of therapeutic doses was doubtful. Any theory based on these results which suggests that digitalis acts by lowering the K/Ca ratio appears to be untenable, since Calhoun and Harrison also found, in fatal human cases with cardiac failure, that the myocardium of the dilated chambers was low in K. Confirmation of this effect of toxic doses has been obtained (Wood and Moe, 1938 ; Hagen, 1939 ; Wedd, 1939 ; Boyer and Poindexter, 1940) though these authors find the effect of therapeutic doses on K content is either negligible or else is to increase it. K loss by digitalis action is also recorded from frog skeletal muscle (Cattell and Goodell, 1937), while further evidence of an effect of digitalis on K metabolism in general is given by Zwemer and Lowenstein (1940), who found that digitalis lowers the plasma K and prolongs life in adrenalectomized animals, thus calling attention to the chemical similarity between the digitalis bodies, especially digitoxigenin, and cortin. Dorfman (1940), however, using adrenalectomized mice, was unable to demonstrate any cortin-like activity of strophanthin.

Therapeutic advantage has been taken of the antagonism between potassium and digitalis (Sampson, Albertson and Kondo, 1943) in order to alleviate the cardiac effects of overdosage of digitalis by giving oral doses of potassium acetate. No relief of nausea and vomiting was obtained, but visual disturbances disappeared.

The most recent publications concerning digitalis and heart biochemistry (Chen and Geiling, 1947) show that toxic doses of digitalis diminish cardiac

adenosine triphosphate (ATP), phosphocreatine and adenylic acid, while therapeutic doses have no such effect, and in decompensated hearts the re-synthesis of these three substances was hastened by digitalis administration (Weicker, 1935). These facts, together with the observation that the isolated perfused rabbit heart in systolic contracture from digitalis can be temporarily restored by ATP (Chen and Geiling, 1946), tempt speculation that the K/Ca effects of the cardiac glycosides may exert an influence on the intricacies of the higher energy-liberating phases of the chemical reactions concerned with muscle contraction.

The experiments now described give results which agree with those of the majority; increased calcium potentiates, and increased potassium antagonizes digitalis glycosides. The quantitative aspect takes the matter further, demonstrating the relative importance of the absolute concentration of potassium in the cardiac action of the glycosides, while the failure to protect pigeons against their emetic effect suggests that different biochemical mechanisms are concerned in the cardiac and emetic actions at least.

The ability of potassium salts not only to delay the toxic action of digitalis, but also to remove toxic effects when already developed is not generally known. Sampson and his colleagues (1943) gave 5–10 g. potassium acetate by mouth as a 25 per cent solution to a series of 14 patients in whom digitalis had produced ectopic beats which were recorded by the electrocardiograph. Only one dose was given on any one day. The authors followed the rise in serum potassium and observed the disappearance of the ectopic beats. This occurred in every patient and outlasted the change in serum potassium. The observations described in this paper add support to these findings and suggest that they are due to the interplay of potassium and digitalis in the heart muscle itself.

Summary

1. The LD of ouabain by intravenous infusion in physiological saline was determined on 34 rabbits under urethane.

2. The effect of $CaCl_2$ and of KCl on the LD of ouabain was observed by adding them to the perfusion fluid: 1, 2, and 3 per cent solutions of $CaCl_2$ decreased the LD of ouabain by 17, 36, and 54 per cent respectively; 0.5, 1, and 2 per cent solutions of KCl increased the LD by 29, 47, and 59 per cent respectively.

3. Survival times were observed of 36 Langendorff rabbit hearts perfused at 36° C. with Locke's solution containing 0.4 μg. ouabain/ml. The calcium or potassium concentration was varied in different experiments.

4. Increased calcium or decreased potassium shortened survival time, while decreased calcium or increased K lengthened it.

5. The effects of altering the potassium were greater than those of corresponding changes in calcium. Halving the $CaCl_2$ prolonged mean survival time by 6 min.; increasing the KCl by 50 per cent prolonged it by 133 min.

6. In a group of 20 pigeons injected via the wing vein with ouabain (15 μg./300 g. pigeon) on two occasions, 22 out of 40 injections caused vomiting. On two other occasions with the same dose of ouabain plus KCl (6 mg./300 g. pigeon), 19 out of 40 injections caused vomiting. The difference is not significant.

I wish to thank Prof. Burn for the guidance and advice he has given throughout.

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THE BLOCKING EFFECT OF BIS-TRIETHYL-AMMONIUM SALTS ON TRANSMISSION IN THE PERFUSED SUPERIOR CERVICAL GANGLION OF THE CAT

BY

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(Received June 9, 1947)

The action of the tetra-ethylammonium ion on the circulatory system was described by Burn and Dale (1914) as a paralysing action on sympathetic ganglia resembling that of nicotine; the same type of action was observed in a series of triethylalkylammonium salts by Hunt (1925–6). The action of tetra-ethyl-ammonium bromide on the circulatory system as well as on the sympathetic ganglion has recently been analysed by Acheson *et al.* (1946). They concluded that the predominant effect of the tetra-ethylammonium ion was a block of transmission across autonomic ganglia and that this was sufficient to explain the vaso-depressor effect. Dr. H. R. Ing suggested to us that it might be worth investigating the effect on ganglionic transmission of bis-triethylammonium salts of the general formula $[Et_3N(CH_2)_nNEt_3]X_2$, where X is the anion; such salts might be expected to have a blocking action on transmission in virtue of the triethylammonium groups and their potency might be expected to vary with the length of the polymethylene chain. Four bis-triethylammonium salts have been examined: ethylene bis-triethylammonium bromide (BTE2), prepared by Dr. H. R. Ing; and the trimethylene-, pentamethylene-, and decamethylene-bis-triethylammonium bromides, denoted by BTE3, BTE5, and BTE10 respectively, which were prepared by Mr. R. B. Barlow.

METHOD

Cats were anaesthetized with pentobarbitone and the superior cervical ganglion was prepared by Kibjakow's method (1933) modified by Feldberg and Gaddum (1934). Warm oxygenated Locke's solution was perfused through a cannula in the carotid artery at a pressure of about 120 mm. of mercury and the venous outflow from the ganglion was collected. The pre-ganglionic fibres to the superior cervical ganglion were stimulated maximally at a rate of 8 stimuli per second, for a period of 15 sec. An interval of 3 min. was allowed between each stimulation. The contraction of the nictitating membrane was

*Working with a grant from the Spanish Council of Scientific Research.

recorded on a smoked drum by an isotonic lever. The activity of the four bis-triethylammonium compounds was compared with that of tetra-ethylammonium bromide (TE). Each drug was given in 0.1–0.2 ml. Locke's solution and was injected into the arterial cannula 1 min. before the stimulation. A cumulative effect by the drug is very liable to occur when the perfusion rate becomes slow; it was therefore decided to test the drugs in one order and then in the reverse order.

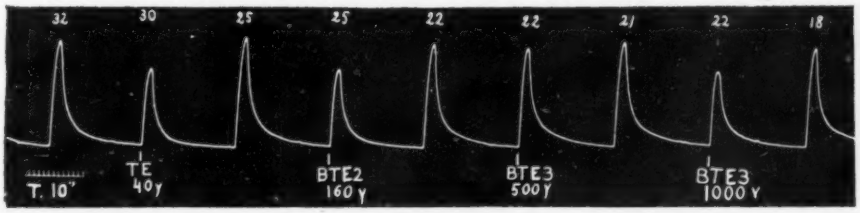

FIG. 1.—Comparison of blocking effect of tetra-ethylammonium bromide (TE) and of two bis-triethylammonium bromides (BTE2 and BTE3) on transmission in sympathetic ganglia. Perfused superior cervical ganglion of the cat. Record of the contractions of the nictitating membrane to maximal preganglionic stimulation. Rate of stimulation 8 per sec. for 15 sec. every minute. Figures above each contraction = outflow in drops per minute.

RESULTS

Fig. 1 shows the effect of BTE2 and BTE3 in comparison with that of TE on the response to preganglionic stimulation in the same preparation. The figures above each contraction of the nictitating membrane represent the venous outflow in drops per minute.

It was found that the blocking effect of the five compounds examined was in the order:

$$\underset{(200)}{BTE10} > \underset{(100)}{TE} > \underset{(33)}{BTE2} > \underset{(5)}{BTE5} > \underset{(4)}{BTE3}$$

The relative potency of these five compounds (according to their weight) has been determined on seven preparations, and, giving TE a value of 100, the mean

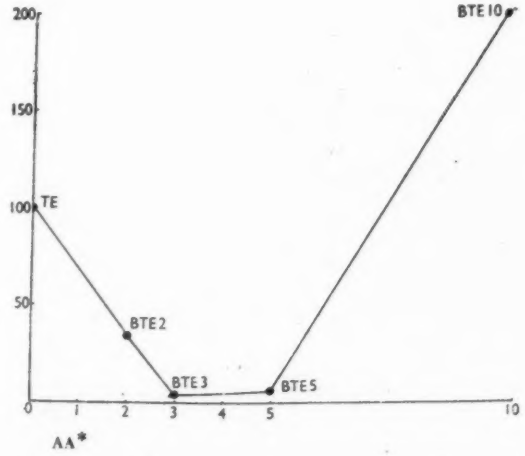

FIG. 2.—The relationship between the activity of tetra-ethylammonium bromide (TE), given a value of 100, and of bis-triethylammonium bromides (BTE2, BTE3, BTE5, and BTE10) in the perfused sympathetic ganglion of the cat. Ordinate: percentage potency. Abscissae: Number of carbon atoms in the polymethylene chain of the molecule.

figures are expressed in brackets. No excitatory action on the ganglion was observed with TE, nor with the four bis-triethylammonium compounds.

Fig. 2 shows the relationship between the activity of these compounds and the number of carbon atoms in the polymethylene chain of the molecule.

Discussion

In their two successive papers, Acheson *et al.* (1946) concluded that the tetra-ethylammonium ion exerts a purely blocking effect on ganglionic transmission. Unlike tubocurarine, the tetra-ethylammonium ion on injection never causes a contraction of the nictitating membrane, nor does it increase the response to electrical stimulation, as intocostrin was shown by Acheson *et al.* to do. It has only a "nicotine-like paralysing" action on the autonomic ganglion. The bis-triethylammonium compounds also showed no excitatory action. No experiments have been made in order to discover whether they have any muscarine actions. In general, the action of these compounds is very similar to that of tetra-ethylammonium salts. As shown in Fig. 2, lengthening of the carbon chain between the two onium ions leads to a decrease in activity until the three carbon chain compound (BTE3) is reached; with a further lengthening of the carbon chain there occurs an increase of the blocking effect, and the 10 carbon chain compound (BTE10) is twice as strong as TE.

Summary

Four bis-triethylammonium compounds have been tested on the perfused superior cervical ganglion of the cat. They have no stimulating action, but they paralyse ganglionic transmission. There is a relationship between the relative activity of these compounds and the number of carbon atoms in the polymethylene chain of their molecules. The decamethylene-bis-triethyl-ammonium bromide (BTE10) was found to be the most potent compound.

We wish to thank Prof. J. H. Burn and Dr. Edith Bülbring for their guidance and encouragement in this work.

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THE PERSISTENCE IN THE BLOOD STREAM OF SOME ANALOGUES OF SULPHADIMETHOXYPYRIMIDINE

BY

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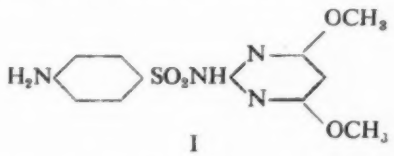
(Received May 14, 1947)

An account has already been given of the properties of sulphadimethoxypyrimidine (I), which displays marked persistence in the blood stream after oral or parenteral administration (Gage *et al.*, 1947).



I

The availability of some of its homologues (Rose and Tuey, 1946) prompted a comparative examination of their persistence; further compounds were specially prepared by Drs. F. L. Rose, E. H. Hoggarth, and E. H. P. Young. Twenty compounds in all were examined in mice for absorption and persistence. The majority were also tested as antibacterial agents; an account of their tuberculostatic activity *in vitro* and *in vivo* will be published separately (Hoggarth, Young, and Martin, 1948). No compound appeared likely to have marked therapeutic value. Three (III, VII, and XVI), as well as sulphadimethoxypyrimidine itself, had previously been examined by van Dyke *et al.* (1945); their findings in general parallel those described here.

Experimental Section

The standard techniques used have been described in previous publications (Rose and Spinks, 1946, 1947; Gage *et al.*, 1947). Each compound was administered orally to a group of three mice, as a 1 per cent (w/v) solution of the sodium salt, or as a 1 per cent (w/v) dispersion, in doses of 250 mg./kg. It was then estimated in pooled tail blood, at standard intervals after dosing, by the micro-method of Rose and Bevan (1944). At least six groups of three mice were used for each compound (except XXI). No statistical comparison was attempted, because many compounds were so highly persistent that the maximum concentration was difficult to determine accurately in individual experiments. Values of maximum

concentration (Max.), the time after dosing at which this was attained (t max.), and the persistence in the blood (expressed as the time (C7) taken for the concentration at 7 hours to fall to two-thirds of that value), were read from the mean blood concentration-time curves.

TABLE

COMPARISON OF ANALOGUES OF SULPHADIMETHOXYPYRIMIDINE

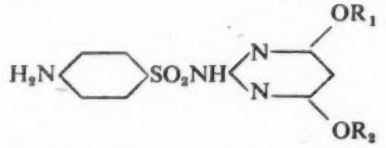
Group	Compound No.	Max. blood concentration		C7 (hours)	Number of mice
		mg./100 ml.	Time (min.)		
A	I	14.4	210	16.5	66
	II	13.8	100	15.3	18
	III	11.8	90	13.0	18
	IV	5.8	540 (?)	>17.0	24
	V	2.2	180	7.8	21
B	VI	5.7	180	10.4	21
	VII	2.1	210	17.0	18
C	VIII	4.6	150	11.0	39
	IX	2.3	210	10.5	18
	X	5.7	270	9.0	30
D	XI	12.6	90	4.7	18
E	XII	24.7	90	7.7	30
	XIII	5.7	120	8.6	27
	XIV	5.9	40	6.0	18
F	XV	13.8	60	7.5	18
	XVI	18.6	65	5.5	18
	XVII	13.7	45	7.6	18
G	XVIII	7.0	40	7.1	18
	XIX	3.5	40	*	18
	XX	0.9	40	*	21
	XXI	5.1	150	†	6

* Disappears very rapidly from the blood. † Disappears rapidly from the blood.

Results and Discussion

Compounds have been classified on the basis of their chemical structure into seven groups, each of which is considered separately; the characteristic values obtained from the mean concentration-time curves are given in the Table.

Group A. 2-*Sulphanilamido*-4 : 6-*di*-n-*alkoxypyrimidines*



Compound No.	I	II	III	IV	V
R_1	CH_3	CH_3	C_2H_5	n-C_3H_7	n-C_4H_9
R_2	CH_3	C_2H_5	C_2H_5	n-C_3H_7	n-C_4H_9

The mean concentrations of these compounds (except II) found in the blood of mice at intervals after the oral administration of 250 mg./kg., are recorded in Fig. 1 ; the characteristic values obtained from the mean curves are compared in the Table. Compounds I–IV are highly persistent ; the di-*n*-butoxy homologue (V) is fairly persistent. The other main difference between the five compounds is in maximum blood concentration, which falls with increasing molecular weight ; this effect can probably be ascribed mainly to reduced solu-

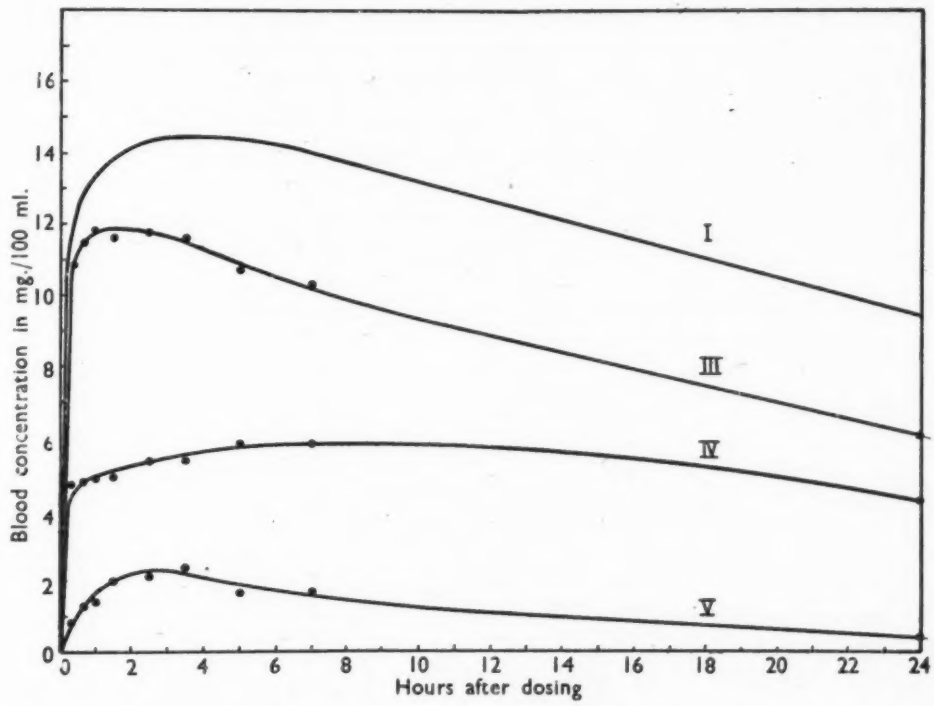
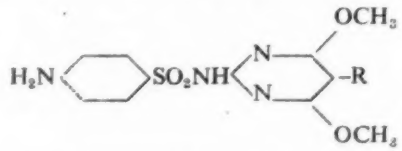


FIG. 1.—Blood concentrations in mice of sulphadimethoxypyrimidine (I), sulphadiethoxypyrimidine (III), sulphadi-*n*-propoxypyrimidine (IV) and sulphadi-*n*-butoxypyrimidine (V).

bility of the higher homologues, which would result in a decreased concentration gradient between lumen and blood stream, and therefore in decreased speed and extent of absorption. The solubility data reported by van Dyke *et al.* (1945) for I and III, and an extensive series of related compounds, support this view. The practical importance of the effect is pointed out by Hoggarth *et al.* (1948), who show that *in vitro* tuberculostatic activity increases with increasing molecular weight. It is probably the accompanying decrease in blood concentration which prevents the higher homologues from showing marked therapeutic action *in vivo*.

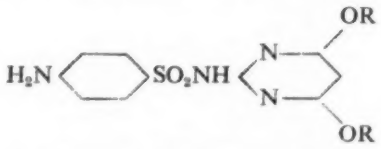
Group B. 2-*Sulphanilamido*-5-*alkyl*-4 : 6-*dimethoxypyrimidines*



Compound No.	VI	VII
R	CH_3	C_2H_5

These two compounds are both highly persistent (see Table), but attain lower maximum blood concentrations than the parent compound. The reduction in maximum concentration is greater than when the additional carbon atoms are substituted in the alkoxy group—i.e., VI and VII give lower concentrations than their isomers II and III. This was unexpected, since in other groups of compounds it has been found that the distribution of a given number of methylene groups between three, instead of two, normal alkyl radicals leads to increased blood concentrations. The effect may be due to the exceptionally low solubility of this type (van Dyke *et al.*, 1945 ; Rose and Tuey, 1946).

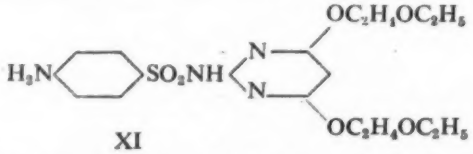
Group C. Branched chain sulphadialkoxy derivatives



Compound No.	VIII	IX	X
R	*iso*-C_3H_7	*iso*-C_4H_9	*sec*-C_4H_9

The characteristic values of the three compounds are summarized in the Table. They are all highly persistent, the butoxy compounds somewhat less so than the *iso*propoxy compound (cf. Group A). VIII and IX resemble their *normal* isomers (IV and V) in maximum blood concentration ; X gives a much higher maximum concentration than its isomers V and IX. In some other homologous series, e.g., of sulphones, it has been observed that compounds containing branched alkyl chains give much higher blood concentrations than their isomers containing straight chains. It is hoped to describe examples in future publications.

Group D. 2-*Sulphanilamido*-4 : 6-*diethoxyethoxypyrimidine*

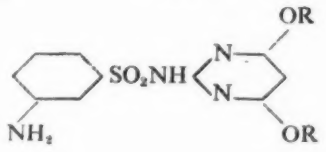


XI

The ethoxyethoxy compound (see Table) has lost much of the persistence characteristic of the other dialkoxypyrimidines, and gives much higher blood

concentrations. It should, of course, be compared with its closest analogue the di-*n*-butoxy derivative (V). The methoxyethoxy homologue was examined by van Dyke *et al.* They found that it was well absorbed, but of low persistence. Its solubility (4 mM./l. at *p*H 6.5 and 37° C.) was five times that of the dimethoxy compound (0.8 mM./l.), and about thirty times that of the diethoxy compound (0.12 mM./l.). It may be assumed that the facile absorption of these alkoxyalkoxy compounds is related to their relatively high solubility, but a different effect must cause the low persistence. The relatively low persistence of the di-*n*-butoxy compound (Group A) suggests that this effect might be connected with steric hindrance; possibly, the bulky alkoxy groups of these compounds hinder their access to some other molecule with which they must be associated to exhibit marked persistence.

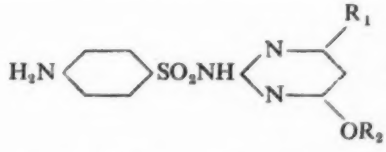
Group E. 2-*Metanilamido*-4 : 6-*dialkoxypyrimidines*



Compound No.	XII	XIII	XIV
R	CH_3	*n*-C_3H_7	*iso*-C_3H_7

The metanilamides are compared in the Table. Reference should also be made to the properties of the corresponding *para* isomers. Each metanilamide differs from its *para* isomer in giving higher maximum blood concentrations, and in disappearing more rapidly from the blood stream. Nevertheless, all three compounds are fairly persistent, approximately of the same order as sulphamerazine (Rose and Spinks, 1946).

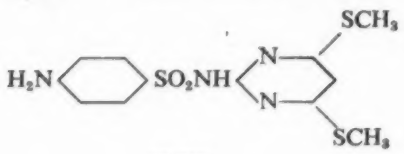
Group F. 2-*Sulphanilamido*-4-*alkoxypyrimidines*



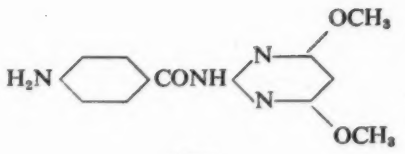
Compound No.	XV	XVI	XVII
R_1	CH_3	CH_3	*iso*-C_3H_7
R_2	H	CH_3	CH_3

The results with these three compounds are given in the Table. They are all fairly persistent, approximately of the same order as the metanilamides (Group E) and sulphamerazine, but less so than the dialkoxy derivatives. The mean curve for XVII is given in Fig. 2.

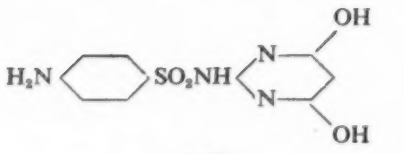
Group G. Miscellaneous compounds



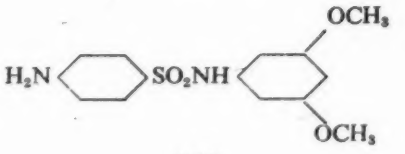
XVIII



XIX



XX



XXI

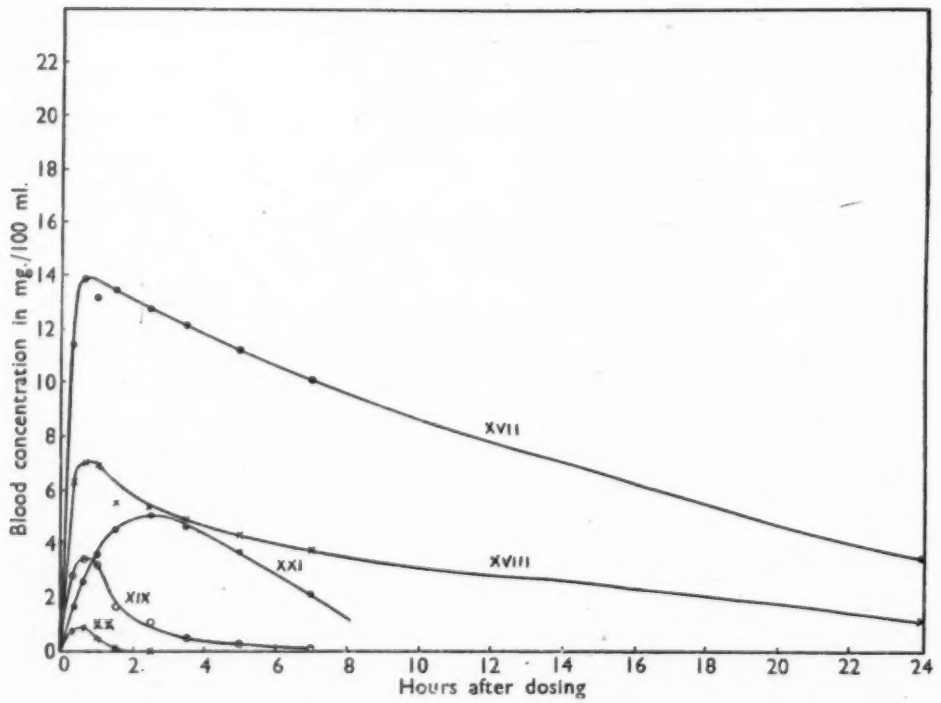


FIG. 2.—Blood concentrations in mice of sulphamethyl*iso*propoxypyrimidine (XVII), sulphadimethylmercaptopyrimidine (XVIII), 2-*p*-aminobenzamido-4 : 6-dimethoxypyrimidine (XIX), sulphadihydroxypyrimidine (XX), and 3 : 5-dimethoxy-1-sulphanilanilide (XXI).

These compounds show striking differences from those previously described (Fig. 2 ; Table). The dimethylmercapto analogue of sulphadimethoxypyrimidine is less persistent than the latter, and gives a much lower maximum concentration. The other three compounds disappear very rapidly from the blood stream. The low concentrations they attain may be associated with this rapid disappearance

from the blood rather than with poor absorption. They clearly show no resemblance whatsoever to sulphadimethoxypyrimidine, although each retains certain structural features of the latter.

Considering the results as a whole, it is clear that marked persistence in the blood stream is conferred by the presence in the sulphapyrimidine molecule of two alkoxy groups in positions 4 and 6 of the pyrimidine ring. All modifications of this structure result in reduction of persistence, including removal of one alkoxy group, transfer of the *para* amino group to the *meta* position, substitution of a benzene ring for the pyrimidine ring, or substitution of a carboxamide group for the sulphonamide group. The persistence of the dialkoxy compounds appears to fall with increasing molecular weight, although one compound (IV) is a marked exception to this rule.

Although the precise nature of the physical and physiological factors which confer persistence on a compound is at present unknown it seems probable that they include a high degree of binding to the plasma proteins and a high degree of tubular reabsorption (Fisher *et al.*, 1943 ; Beyer *et al.*, 1944 ; Earle, 1944 ; van Dyke *et al.*, 1945). The strength of the bond uniting drug to protein may also be of importance (Gregerson and Rawson, 1943 ; Rawson, 1943). Sulphadimethoxypyrimidine and some related compounds have been shown to be extensively bound to plasma proteins (van Dyke *et al.*, 1945 ; Gage *et al.*, 1947), but no information is available on the other important factors. A high degree of protein binding alone would not necessarily result in high persistence, absorption by the tubules being of equal or greater importance (Fisher *et al.*, 1943 ; Lundquist, 1945). Besides these factors, which influence the rate of excretion of a compound by the kidney, and others, less adequately investigated, which influence the rate of excretion into the intestine (*cf.* Silber and Clark, 1946), diazotizable amines are also removed from the blood stream by conversion to non-diazotizable or rapidly excreted metabolites, such as acetyl derivatives, sulphates or glucuronides. It is improbable that the persistence, even of closely related compounds, is uniformly affected by such metabolic processes.

The complexity of all these mechanisms, which influence persistence, is such that speculation on the nature of the correlation between structure and persistence is hardly justifiable. However, it is clear that the correlation is a very delicate one. It had been hoped that 4 : 6-dimethoxypyrimidine, or even *m*-dimethoxybenzene, might behave as a "conductophoric" group, and confer persistence on any molecule containing it, in the same manner as the dialkylamino-alkylamino chain of mepacrine, pamaquin, and 3349 (Magidson *et al.*, 1934, 1936 ; Spinks and Tottey, 1946), or the biguanide chain of paludrine and related drugs (Spinks, 1946, 1947), has been presumed to confer favourable pharmacological properties on the antimalarial containing it. The reduced persistence of compounds XII, XIII, XIV, XIX, and XXI strongly suggests that the introduction of 1 : 3-dimethoxy groups into a nucleus other than pyrimidine, or the combination of

4 : 6-dimethoxypyrimidine as a " conductophoric " group with a " toxicophoric " group other than sulphanilamide would be unlikely to confer high persistence on the resulting molecule.

Summary

The absorption and persistence in mice of twenty compounds related to sulphadimethoxypyrimidine have been described. High persistence is a property of 2-sulphanilamidopyrimidines carrying dialkoxy groups in positions 4 and 6. Removal of one alkoxy group, transfer of the *p*-amino group to the *meta* position, substitution of a benzene ring for the pyrimidine ring, or substitution of a carboxamide group for the sulphonamide group, results in reduced persistence. In each homologous series examined, maximum blood concentration fell with increasing molecular weight.

The author wishes to thank Miss E. France for technical assistance.

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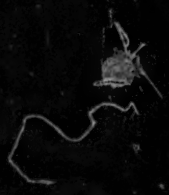

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